# INFLUENCE OF GENOME SPECIFIC GRANULE-BOUND STARCH SYNTHASE I (GBSSI/ WAXY) ON STARCH COMPOSITION, STRUCTURE AND *IN VITRO* ENZYMATIC HYDROLYSIS IN WHEAT (*Triticum aestivum* L.)

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# ABSTRACT

Wheat grain quality and consumption is influenced by its constituents structure and concentrations. In the first part of the dissertation, six Canadian bread wheat cultivars; four (CDC Teal, AC Superb, AC Barrie, AC Splendor) belonging to the Canada Western Red Spring (CWRS), and two (AC Foremost, and AC Crystal) to the Canada Prairie Spring Red (CPSR) market classes were characterized for the relationship between their starch constituents and starch *in vitro* enzymatic hydrolysis. CPSR cultivars with relatively longer amylopectin chains of DP 37-45, reduced chain lengths of DP 15-18, and a low volume percent of small C-type starch granules, had reduced starch *in vitro* enzymatic hydrolysis rates. In the second part of the dissertation, near-isogenic wheat lines differing at the *Waxy* locus were analyzed for the influence of genome-specific granule-bound starch synthase I (GBSSI/Waxy; Wx-A, Wx-B, Wx-D) on starch composition, structure and starch *in vitro* enzymatic hydrolysis. Amylose concentration was more severely affected in genotypes with GBSSI missing from two genomes (double nulls) than from one genome (single nulls) of wheat, indicating dosage dependent amylose synthesis. Subtle differences in amylopectin chain length distribution were observed among non-waxy, partial and completely waxy starches, suggesting a non-limiting role of genome-specific GBSSI for amylopectin synthesis. A suppressive role of Wx-D on the short chain phenotype of wheat amylopectin was observed. In addition, Wx-D increased the volume percentage of large A-type starch granules and reduced starch hydrolysis index. Thus, among the waxy isoproteins, Wx-D might be the major contributor for reducing the rate of *in vitro* starch enzymatic hydrolysis in wheat. In the third part of the dissertation, endosperm starch's physicochemical properties and structure during grain development in wheat waxy-null genotypes were analyzed. The study was conducted with pure starch isolated from wheat grains at 3-30 days post anthesis (DPA), at three day intervals. Changes in amylopectin structure were observed until 12 DPA, suggesting the formation of a basic amylopectin skeleton by this stage. A differential influence of waxy isoproteins on amylopectin structure formation has been suggested, with Wx-B and Wx-D affecting short glucan chains of DP 6-8 at 3 and 6 DPA, Wx-A being effective at 9 and 12 DPA, and Wx-D affecting DP 18-25 chains from 18-30 DPA.

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| | | |
|---|---|---|
| 3PGA | : | 3-Phosphoglyceric acid |
| AFM | : | Atomic force microscopy |
| AGPase | : | ADP-glucose pyrophosphorylase |
| AMG | : | Amyloglucosidase |
| APTS | : | 8-Aminopyrene 1,2,6-trisulfonate |
| CPSR | : | Canada Prairie Spring Red |
| CWRS | : | Canada Western Red Spring |
| DBE | : | Debranching enzyme |
| DMSO | : | Dimethyl-sulfoxide |
| DP | : | Degree of polymerization |
| DPA | : | Days post anthesis |
| F6P | : | Fructose-6-phosphate |
| FACE | : | Fluorophore assisted capillary electrophoresis |
| G1P | : | Glucose-1-phosphate |
| G6P | : | Glucose-6-phosphate |
| GBSS | : | Granule-bound starch synthase |
| GI | : | Glycemic index |
| GOPOD | : | Glucose oxidase/peroxidase |
| GWD | : | Glucan water dikinase |
| HI | : | Hydrolysis index |
| HP-SEC | : | High-performance size exclusion chromatography |
| ISA | : | Isoamylase |
| LDA | : | Limit dextrinase |
| MOS | : | Malto-oligosaccharides |
| PDI | : | Polydispersity index |
| Pho1 | : | Phosphorylase-1 |
| PWD | : | Phosphoglucan water dikinase |
| RDS | : | Rapidly digestible starch |
| RS | : | Resistant starch |
| SBE | : | Starch branching enzyme |
| SDS | : | Slowly digestibility starch |
| SEM | : | Scanning electron microscopy |
| SS | : | Starch synthase |
| SuSy | : | Sucrose synthase |
| TGW | : | Thousand grain weight |
| Wx | : | Waxy |

# 1. INTRODUCTION

## 1.1 Background and rationale

Wheat is the third largest cereal grown after maize and rice, and supplies much of world's food supply. Its adaptability to varying environmental conditions and diverse end-uses has made it one of the major crops of the world. Canada ranks seventh in the world's wheat production (http://www.fas.usda.gov/psdonline/circulars/grain.pdf). Saskatchewan accounts for ~50% of the total wheat grown in Canada. Cytogenetically, wheat is an allohexaploid (*Triticum aestivum* L., 2n = 6x = 42, AABBDD). It originated initially by hybridization of its first two diploid progenitors; *Triticum urartu* (donor of A genome) and *Aegilops speltoides* (donor of B genome), which gave rise to tetraploid durum wheat *Triticum turgidum* (BBAA). The tetraploid wheat then hybridized with *Aegilops tauschii* (donor of D genome) to form bread wheat *Triticum aestivum* (BBAADD) (Jauhar, 2007).

Wheat utilization depends upon the relative concentrations of main storage components of the grain. A typical wheat grain stores carbohydrates principally in the form of starch, which is utilized by the plant during germination and seedling growth. Starch is a glucan homopolymer synthesized from photosynthetically derived carbon. Biochemically, starch is composed of two kinds of molecules, (i) amylose, which is essentially linear, scarcely branched (one branch per ~ 1000 residues) and occupies one-quarter of starch, and (ii) amylopectin, which is highly branched (4-5%) and occupies three-quarters of starch (Chibbar *et al*., 2007). The ratio of amylose: amylopectin determines the end use of starch. Higher amylose concentration leads to Resistant Starch (RS) i.e. starch which resists digestion in the small intestine of healthy people (Ito *et al*., 1999; Sajilata *et al*., 2006). Recently it has been reported that alteration in amylopectin chain length distribution also affects digestibility (Ao *et al*., 2007; Shu *et al*., 2007). Resistant starch offers several positive physiological effects on human body such as reduction in glycemic index, cardiovascular diseases, production of butyrate which acts against colorectal cancer, prevention of fat accumulation and better absorption of minerals like calcium and iron (Mentschel and Claus, 2003; Chibbar *et al*., 2010).

Starch synthesis is a complex process and involves multiple enzymes. Initial reaction of starch synthesis is carried out by ADP-glucose pyrophosphorylase (AGPase), which catalyzes

the formation of ADP-glucose from glucose-1-phosphate and ATP. Amylopectin synthesis involves an array of enzymes with various isoforms such as starch synthases (SSI, IIa, IIb, III, IV) for glucan chain elongation, starch branching enzymes (SBEI, IIa, IIb) for introducing branches and starch debranching enzymes (ISA, LDA) for trimming of external glucan chains (James *et al*., 2003; Kötting *et al*., 2010; Tetlow, 2011). Amylose synthesis, on the other hand, is carried out by a single enzyme called granule-bound starch synthase I (GBSSI/ Waxy), encoded by the '*waxy'* loci in wheat. GBSSI has also been postulated to have a role in elongation of extra-long glucan chains of amylopectin (Ral *et al*., 2006).

Since bread wheat is an allohexaploid, hosting three genomes, each protein would be present in three copies, one originating from each genome. Hence three forms of GBSSI are present in wheat i.e., Wx-A, Wx-B and Wx-D. It has been reported earlier that the contribution of the three waxy proteins towards amylose synthesis varies in the order Wx-B ≥ Wx-D > Wx-A (Miura *et al*., 1999) or, Wx-D > Wx-B > Wx-A (Debiton *et al*., 2010). Moreover, different combinations of the waxy proteins have been shown to result in different amounts of starch constituents, for instance amylose concentration (Demeke *et al*., 1999; Kim *et al*., 2003). A report has also implicated that deficiency of GBSSI alters the amylopectin chain length distribution in wheat (Li Chun-Yan *et al*., 2007). Therefore, we hypothesize that starch biosynthetic enzymes encoded by the three wheat genomes have differential enzymatic activity and differently influence starch biochemical and functional characteristics like starch digestibility.

Starch granules of different shapes and sizes exist in the endosperm of a wheat grain. Mature endosperm of wheat consists of two or three types of starch granules with variable diameters; large disc-shaped A-type starch granules (> 15 μm), small spherical B-type starch granules (5-15 μm) and few reports suggest the occurrence of very small C-type starch granules (< 5 μm) (Bechtel *et al*., 1990). Different types of starch granules are formed at different stages during wheat grain development. A-type starch granules appear four days after anthesis and continue to increase in size throughout the grain filling period, while B-type starch granules are initiated 12-14 days after anthesis and remain considerably smaller. C-type starch granules start appearing from 21 days after anthesis (Rahman *et al*., 2000). Different shapes of starch granules have been postulated to contain different arrangements of amylose and amylopectin (Ao and Jane, 2007). Hence, we speculate that a change in starch granule morphology during wheat grain

development is accompanied with a change in molecular organization of glucans within the starch granule.

In this thesis, waxy-null near-isogenic wheat lines have been studied to understand the influence of genome-specific GBSSI on starch characteristics and its relation with *in vitro* enzymatic starch hydrolysis. The later part of the thesis deals with the study of endosperm starch characteristics during wheat grain development.

## 1.2 Hypotheses

(a) Starch hydrolysis is affected both by starch composition and amylopectin structure.
(b) Starch biosynthetic enzymes encoded by each of the three wheat genomes differentially affect starch accumulation and its properties.
(c) Change in starch granule morphology is accompanied by change in molecular organization of glucans within a starch granule.

## 1.3 Objectives

(a) Characterize selected Canadian bread wheat cultivars for starch structure differences and its influence on starch *in vitro* enzymatic digestibility.
(b) Demonstrate the effect of genome specific GBSSI on starch characteristics and its influence on starch *in vitro* enzymatic hydrolysis.
(c) Analyze changes in starch granule structure and characteristics during wheat grain development.

# 2. LITERATURE REVIEW

## 2.1 Wheat

Wheat, *Triticum aestivum* L., belongs to the tribe *Triticeae* and the genus *Triticum* of the grass family *Poaceae* (Peterson, 1965). Wheat is the second largest cereal crop in the world after maize with annual production of 701 million metric tons in 2013 (USDA, 2013). Leading producers of wheat are the European Union, China, India, United States, and Russia which contribute to more than 70% of the world wheat production (USDA, 2013).

### 2.1.1 Evolution of hexaploid wheat

Bread wheat (*Triticum aestivum* L.) has a complex genome consisting of three related genomes that are derived from three different diploid species. It is called an allohexaploid (*allo*, from Greek, meaning "different"). Genomic studies by various workers have estimated the time of wheat speciation. Historically, diploid progenitors of allohexaploid wheat diverged from a common ancestor around 2.5 – 4.5 million years ago; allotetraploid wheat was formed about 300,000 – 500,000 years ago (Huang *et al*., 2002); and allohexaploid wheat was formed only about 10,000 years ago (Feldman, 2001). Wheat being one of the foremost crops of the world has been domesticated in the Fertile Crescent in the region west of Diyarbakir in southeastern Turkey (Heun *et al*., 1997; Luo *et al*., 2007). With the expansion of agriculture, domesticated einkorn (*T. urartu*, genome AA) and emmer (*T. turgidum*, genomes BBAA) spread through Asia, Europe and Africa. During the northeast expansion of emmer cultivation, sympatry with *Aegilops tauschii* (genome DD) resulted in the formation of hexaploid wheat/ dinkel (genomes BBAADD) (Dvořák *et al*., 1998).

Cytogenetically, bread wheat is an allohexaploid species (2n = 6x = 42, BBAADD). It originated from two hybridization events and three diploid progenitors. First step involved the hybridization between *Triticum urartu* (donor of A genome, Chapman *et al*., 1976) and a yet undiscovered *Aegilops* species closely related to *Ae. speltoides* (donor of B genome) forming allotetraploid wheat, *Triticum turgidum* (genomes BBAA). The allotetraploid wheat hybridized with *Ae. tauschii* (donor of D genome, McFadden and Sears, 1946) to form the present allohexaploid wheat, *Triticum aestivum* (genomes BBAADD) (Figure 2.1).

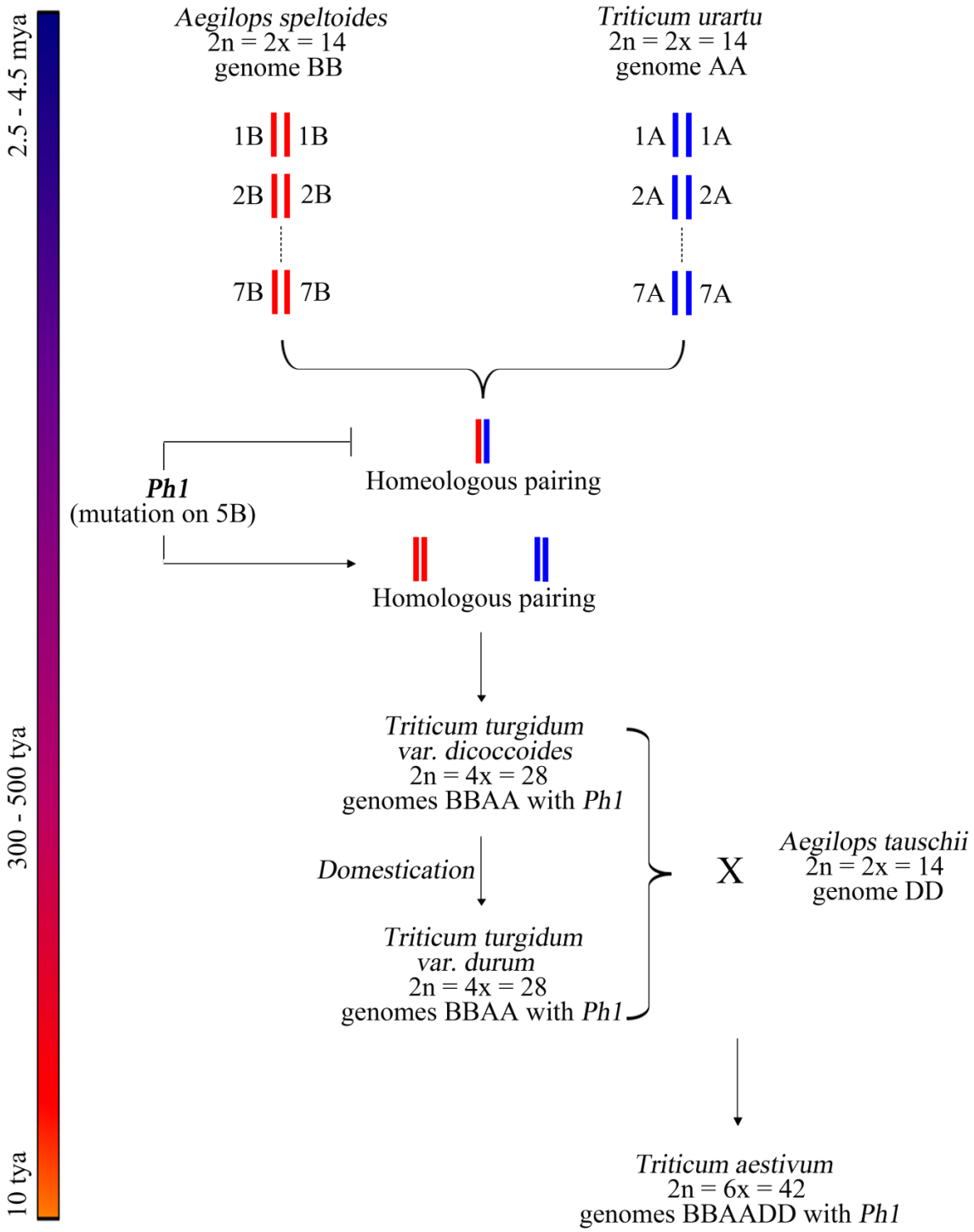



Figure 2.1 Evolution of allohexaploid wheat

Abbreviations: mya – million years ago, tya – thousand years ago. Chromosomes depicted with the same colour represent homologous chromosomes from one parent. Durum wheat is designated BBAA because *Ae. speltoides* is the considered to be the cytoplasm donor in wheat (Jauhar, 2007).

Genetically, seven pairs of homologous chromosomes from each genome (total 21 pairs) are classified into seven homeologous groups. Each homeologous group contains one pair of homologous chromosomes from A, B and D genomes. For example, homeologous group 1 contains the homologous chromosome pair 1A, 1B and 1D.

Since the corresponding homeologous chromosomes were closely related, there was a high probability of pairing between them. To regulate inter-genomic hybridization, a mutation called *pairing homeologous 1 (Ph1)* arose on the long arm of chromosome 5B at the time of formation of tetraploid wheat. The *Ph1* gene suppresses the pairing of homeologous chromosomes, while promoting the pairing of homologous chromosomes (Figure 2.1); thereby ensuring diploid-like meiosis and disomic inheritance of polyploid wheats (Riley and Chapman, 1958; Jauhar *et al*., 1991). A mutation such as *Ph1* has not been found on corresponding chromosomes 5A and 5D (Sears, 1976).

There are several advantages associated with polyploidy. Wheat consists of three genomes, each adapted to different environments, hence making it more adaptable to a wider range of environmental conditions. For instance, hexaploid *T. aestivum* has broad adaptability to different photoperiod and vernalization requirements; improved tolerance to salt, low pH, aluminium, and frost; better resistance to several pests and diseases; and extended potential to make different food products, as compared to tetraploid wheat (Dubcovsky and Dvořák, 2007; Feldman and Levy, 2012). On the other hand polyploidy has its disadvantages as well, for example polyploidy bottleneck (Stebbins, 1950) where species with less number of contributing plants towards the formation of a polyploidy species, constrains its initial gene diversity. During the formation of hexaploid wheat, few genotypes of *Ae. tauschii* participated in the event, thus narrowing the diversity of D-genome. However, the hexaploid wheat compensated for diversity bottleneck by capturing large proportion of variability of the tetraploid progenitor. In addition, new variations are generated by gene deletions and insertions in the coding or regulatory gene regions, which can be expressed quantitatively considering the polyploid nature of wheat. Synergy between the high mutation rates and the buffering effects of polyploidy makes it possible for polyploid wheat to capitalize on the diversity generated by its dynamic genomes (Dubcovsky and Dvořák, 2007).

### 2.1.2 Wheat in Saskatchewan

Canada is the sixth largest producer of wheat in the world (USDA, 2013). Saskatchewan is the leading wheat producing province in Canada, followed by Alberta, Manitoba, and Ontario (USDA, 2013). Historically, wheat production in Canada started during the early 17$^{th}$ century and in Saskatchewan sometime around 1753 – 1756. Wheat's wide adaptability is reflected by its cultivation over all soil zones across the province. Saskatchewan currently accounts for ~50% of the wheat produced in Canada (http://www.agriculture.gov.sk.ca/Default.aspx?DN=5d77b997-029c-49fa-8d72-8729c8278698); in the province, wheat accounts for 44% of the area seeded to crops.

The Canadian Grain Commission classified the western Canadian wheats into eight milling classes:

1. Canada Prairie Spring Red (CPSR)
2. Canada Prairie Spring White (CPSW)
3. Canada Western Amber Durum (CWAD)
4. Canada Western Extra Strong (CWES)
5. Canada Western Hard White Spring (CWHWS)
6. Canada Western Soft White Spring (CWSWS)
7. Canada Western Red Spring (CWRS)
8. Canada Western Red Winter (CWRW)

Among the eight milling classes of wheat, CWRW is the only one with winter growth habit, others being of spring growth habit and constitute ~98.8% of all the wheat grown in Saskatchewan. Among the spring wheats, CWRS occupies maximum seeded area to wheat in Saskatchewan followed by CPSR/ CPSW, CWES and CWSWS/ CWHWS. On a percentage basis, the area occupied by these wheats is 85.5%, 12%, 1% and 0.05%, respectively of the seeded spring wheat acreage. CWRS wheat is considered high protein premium bread wheat with good baking and milling properties. CPS class was developed as a high yielding, lower protein alternative to CWRS.

The classes mentioned so far are hexaploid species with variable grain composition and end-uses. One class which represents tetraploid wheat is CWAD. Saskatchewan is the major producer of durum wheat and accounts for 83% of Canada's total durum wheat production. CWAD's protein level is similar to CWRS and is primarily used for making pasta. The winter wheat class, CWRW, is characterised by good milling properties.

## 2.2 Wheat grain constituents

A typical wheat grain consists of three parts (Figure 2.2). Bran, which is the outermost layer of the kernel, is rich in vitamins and minerals. Endosperm, represents about 80% of the kernel weight, and primarily consists of starch and to a less extent protein. Germ or embryo lies at one end of the grain and represents only about 2% of the kernel weight. Germ is a rich source of vitamin B and E, oils and natural plant fat.

### 2.2.1 Carbohydrates

Carbohydrates occupy 85% (w/w) of a mature wheat grain. These can be divided into mono-, di-, oligo- and polysaccharides occupying different regions in a wheat grain. Starch constitutes 80% of the grain carbohydrates and is present only in the starchy endosperm. Low molecular mass mono-, di-, oligosaccharides, and fructans (~7%) are present in the aleurone, starchy endosperm and tissues of the embryonic axis. In addition, cell wall polysaccharides, which constitute ~12% of the grain carbohydrates, are found in all tissues.

***Mono-, di-, and oligosaccharides***

In mature grain, small amounts of low molecular mass carbohydrates, which are extractable in water or 80% (w/v) ethanol, are found in the embryo, the aleurone, and the endosperm but are virtually absent from the mature pericarp-seed coat tissues. The low molecular mass carbohydrate fraction includes the reducing aldo/keto-hexose monosaccharides (D-glucose and D-fructose, respectively) and minor amounts of their phosphorylated forms, which are intermediates in carbohydrate metabolism. Sucrose, a non-reducing disaccharide composed of D-glucopyranosyl and D-fructofuranosyl residues, is the most prominent

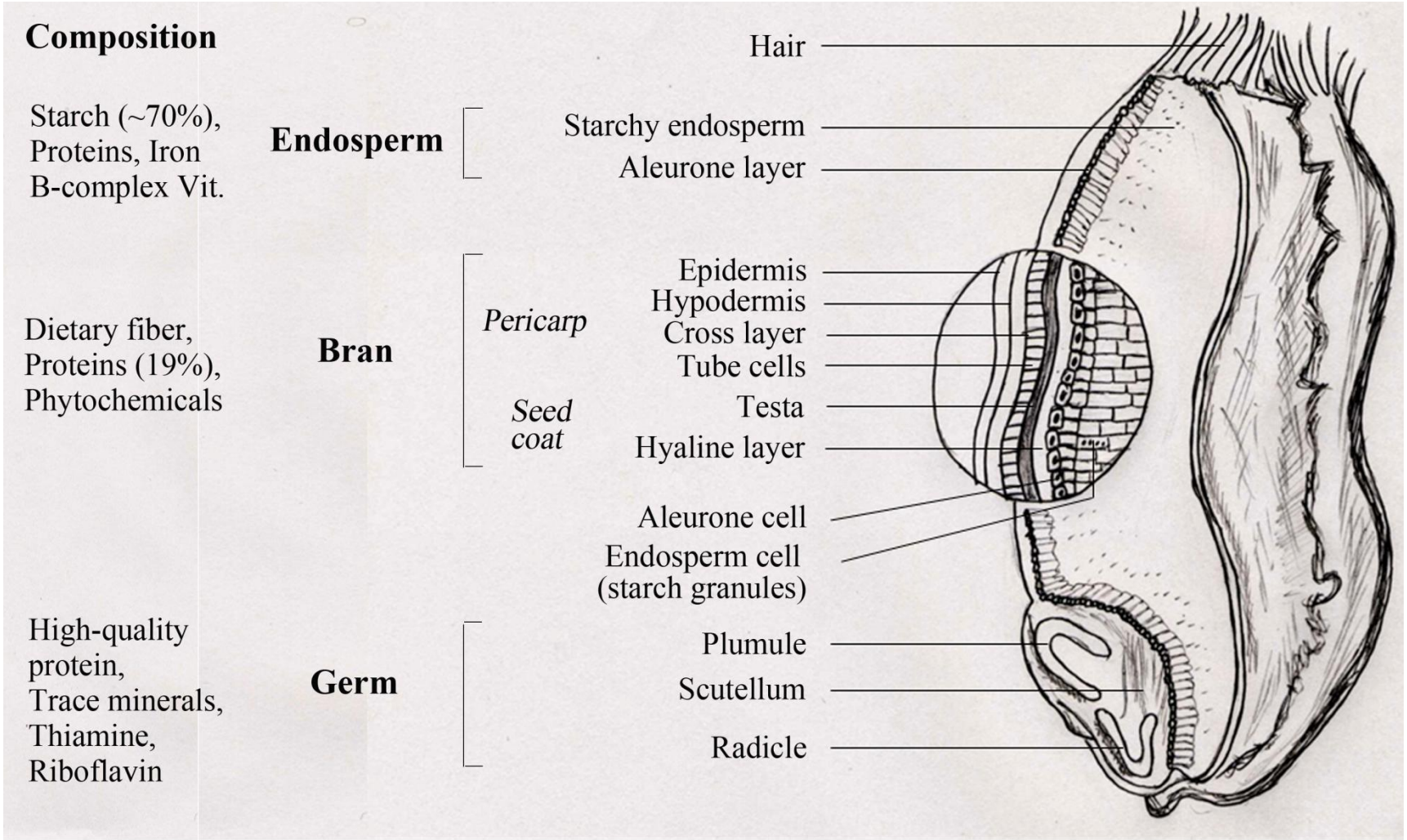



Figure 2.2 Wheat grain showing different components and composition

component of the low molecular mass carbohydrate fraction and is accompanied by its 6-α-O-D-galactopyranosyl derivative, raffinose (Nilsson *et al*., 1986). Sucrose and raffinose are present in both the embryo and scutellar tissues (Dubois *et al*., 1960). In addition, maltose is also present in minor amounts. Fructans are composed of chains of β-D-fructofuranosyl residues in (2, 1)-β-linkage to the fructosyl residue of sucrose, with some branching through (2, 6)-β-linkages. These fructans account for 1.3 – 2.5% (w/w) of mature grain (Henry and Saini, 1989; Knudsen, 1997; Campbell *et al*., 1997) and at maturity are present predominantly in the embryo, but also in the endosperm (Nilsson *et al*., 1986). These mono-, di- and oligosaccharides exist in inter-changeable forms depending on the grain development stage and environmental condition. During grain development, stem fructans are remobilized as sucrose and are moved in the phloem to the developing grain. The level of fructose-containing assimilates like sucrose, show diurnal variation. Sucrose concentration is higher during the day because of active photosynthesis, however, the catabolic products, fructose and glucose, are higher at night (Carman *et al*., 1996; Carman and Bishop, 2004). Under conditions of drought stress, fructans may account for up to 70% of the grain dry matter (Goggin and Setter, 2004).

***Polysaccharides***

*Storage polysaccharides*: Starch – discussed in the later section

*Cell wall polysaccharides*: In wheat grains, the unlignified (primary) walls of cells in the starchy endosperm, aleurone, scutellum, and embryonic axis are characteristically rich in (glucorono)arabinoxylans; and in the case of starchy endosperm and aleurone, mixed-linkage (1, 3; 1, 4)-β-D-glucans. The lignified secondary walls of cells in the pericarp- seed coat of the grain are rich in cellulose (60%) and (glucurono)arabinoxylans (30%), with smaller amounts (1.2%) of mixed-linkage (1, 3; 1, 4)-β-D-glucans (Harris *et al*., 2005).

Properties of storage as well as cell wall polysaccharides affect the human gastrointestinal physiology. Starch can affect the rate of digestion through the gastrointestinal tract to the point of glucose uptake in the small intestine, and determining the amount of starch digestion products passing into the large bowel. The rate of digestion influences the glycemic response of food, whereas the extent of digestion controls the resistant starch content of food (Sajilata *et al*., 2006). Cell wall components of wheat grain tissues- cellulose, arabinoxylans, mixed-linkage (1,3; 1,4)-β-D-glucans and the soluble fructans are important components of fiber in human diets. Humans

produce no autochthonic enzymes that degrade the cell wall polysaccharides or fructans, although they are to a greater or lesser extent fermented by the microbial population in the lower gut, with the production of volatile (short-chain) fatty acids, which are in turn metabolized or absorbed by the mucosal cells (Bugaut and Bentéjac, 1993). Thus, dietary fiber makes a small contribution to digestible energy from food.

### 2.2.2 Proteins

Protein content is an important factor for the bread making process, making it a commercially important component. A positive correlation exists between bread loaf volume and flour protein content (Fifield *et al*., 1950). Biochemically, protein content is determined by calculating the amount of nitrogen in the sample, and multiplying the nitrogen content by a factor of 5.7 for wheat (Mossé, 1990).

Gliadins and glutenins are major proteins in a wheat grain. Monomeric gliadins and aggregative glutenins together form a gluten protein complex in the dough. Although the proportions may vary, the total gluten protein content in Canadian Western Red Spring wheats was shown to be ~10% (Seilmeier *et al*., 1991). Gliadins are classified into Ω-type, α-type, and γ-type gliadins. Glutenins are composed of low molecular weight (LMW) and high molecular weight (HMW) subunits. Glutenins have the ability to form large disulfide-bridged aggregates, which are responsible for the unique properties of the dough (Wrigley, 1996). The very large glutenin polymers contribute resistance to the extension forces of dough-mixing ('springiness'), while gliadin monomers provide a plasticizer function. These properties are utilized in the production of bread, noodles, pasta and other wheat-based foods.

Puroindolines are the grain softness proteins, which determine the grain texture. Patterns of proteins on the surface of water-washed starch granules from a number of soft and hard wheat cultivars were compared using polyacrylamide gel electrophoresis (Greenwell and Schofield, 1986). A 15kDa polypeptide, 'friabilin' was present in the soft cultivars, but not in hard cultivars. Friabilin is comprised of several components, three of which are encoded by genes at the *Hardness* (*Ha*) locus, which is the major locus controlling grain texture in bread wheat (Oda and Schofield, 1997). The *Ha* locus encodes puroindolines (Pins) 'a' and 'b', also called Grain Softness Proteins. Extensive comparisons of sequences of *Pin* genes (located on chromosome

5D) and of textural properties of a large number of wheat properties demonstrated that specific mutations in either the *Pina* or *Pinb* gene were consistently associated with hard endosperm phenotype (Morris, 2002). Pin 'a' was able to bind tightly to both wheat phospholipids and glycolipids, while Pin 'b' bound negatively charged phospholipids tightly but formed loose lipoprotein complexes with glycolipids (Dubreil *et al*., 1997).

Other proteins in the wheat grain include α-amylase inhibitors, lipid transfer proteins, α-globulins, wheat germ agglutinin, ribosome-inactivating proteins, thionins, to name a few. α-amylase inhibitors constitute two-thirds of albumin proteins. These are inactive against endogenous wheat grain enzymes, but inhibit enzymes from other organisms for example, human salivary and pancreatic α-amylases. Initially α-amylase inhibitor proteins were selectively extracted using chloroform-methanol mixtures, and were thus named 'CM' proteins (Salcedo *et al.*, 1978). These exist in multiple forms in the wheat grain, being monomeric (WMAI-1,2), and homodimeric (WDAI-1,2,3). In addition, a tetrameric form also exists, comprising single copies of two subunits (WTAI-CM1/2 and WTAI-CM16/17) and two copies of a third subunit (WTAI-CM3B/D). However individual subunits of the tetramer show little or no activity as monomers (Shewry *et al*., 2009). In a wheat grain, the α-amylase inhibitor proteins are predominantly present in the starchy endosperm (Buonocore *et al.*, 1977). They are synthesized with an N-terminal signal sequence, which indicates that they are initially translocated into the lumen of the endoplasmic reticulum. It is hypothesized that they are further transported to the vacuole with other 'secretory' proteins and ultimately form part of the protein matrix in the mature starchy endosperm cells. Subunits of the α-amylase inhibitor proteins react with IgE from allergic patients with bakers' asthma *in vitro.* Members of this protein family have also been identified as food allergens (Kusaba-Nakayama *et al*., 2001).

Wheat germ agglutinin (WGA), as the name suggests, is present in the wheat germ and possesses the ability to agglutinate. WGA binds N-acetylglucosamine/ chitin, which enables it to bind to the surfaces of several types of cells, to cause agglutination. It thus protects the plants from insects, yeast, and bacteria. In a wheat grain, WGA is present in the embryo, particularly the parts which come in contact with the soil during germination, i.e. embryonic root caps, coleorhizae, outer layers of coleoptiles, radical and scutellum (Mishkind *et al*., 1982). WGA, also called lectins, exist in three forms named isolectin A, B, and D, encoded by the three genomes of hexaploid wheat (Peumans *et al*., 1982). Each isolectin comprises two identical

polypeptide chains of 171 residues, which form dimers of mass about 36 kDa. However, each polypeptide is itself composed of four related domains, which are called 'hevein domains'. A single domain can bind a saccharide but the strength of binding is increased by the interactions of two domains on different monomers, which form a binding site (Wright *et al*., 1991).

### 2.2.3 Lipids

Lipids in a wheat grain or flour, even though being a minor constituent, play a major part in food processing, and product formation (Chung and Ohm, 1997). Lipids occupy 2-3.5% (w/w) of the whole grain and are present on the surface as well as inside of the starch granule. These two groups of lipids differ in extractability from starch granules with common fat solvents. Ether-extractable lipids are considered to be absorbed on the surface of starch granules while lipids existing firmly in starch granules cannot be extracted with diethyl-ether, but can be extracted with hot aqueous alcohol. The surface lipids are mainly triglycerides, followed by free fatty acids, glycolipids and phospholipids and they include those derived from the amyloplast membrane and non-starch sources. The internal lipids of cereal starches are predominantly monoacyl lipids, with the major components being lysophospholipids and free fatty acids. Both surface and internal lipids are present in free-state and linked with starch components, mostly as amylose-lipid inclusion complexes linked via ionic or hydrogen bonds to the hydroxyl groups of the starch components (Morrison, 1988).

Starch lipid contents show significant variations among wheat varieties or classes in relation to amylose content. Higher lipid and amylose contents in A-type and B-type starch granules and total starch were reported for durum wheats than bread wheat (Soulaka and Morrison, 1985).

### 2.2.4 Micronutrients and phytochemicals

Whole wheat grains contain significant amounts of functional, biologically active components (Slavin *et al*., 2001; Jones *et al*., 2002). Micronutrients and phytochemicals are evenly distributed in the wheat grain, primarily in the germ and bran. Most of the antioxidants are contained in bran and germ (Miller *et al*., 2000). Wheat is a good source of several B vitamins, especially thiamin, niacin, vitamin $B_6$ and folate, tocopherols and carotenoids. The

contents of minerals and trace elements in wheat are considerable viz. Ca, Mg, P and K. However, Fe, Zn, Cu and Mn are low. The ash content of wheat can vary from 1.17 – 2.96% (Obert *et al*., 2004). This variation is caused by genotype, wheat class and cultivar, as well as the growing location and year. Great variability in all minerals and trace elements was found (Bálint *et al*., 2001; Akman and Kara, 2003). The general trend was that the diploids had higher mineral and trace element contents than the hexaploids. In addition, wheat grains also contain phytosterols, phenolics, choline and betaine.

Phenolics are referred to the compounds which contain a phenol ring. Based on the number of phenol rings, this group of compounds can be classified into polyphenols and simple phenols. Phenolic compounds constitute a major group of phytochemicals found in plants (Gross, 1981). Examples include amino acids phenylalanine, tyrosine, and tryptophan; the prosthetic groups of many enzymes; the cell wall material lignin; natural and synthetic hormones such as indoleacetic acid (Piironen *et al*., 2009). Certain phenolics may be formed in plants in response to invasion by pathogens or parasites, making the plant resistant to invasion. Physiologically, phenolic acids have diverse functions such as nutrient uptake, enzyme activity, photosynthesis, cell wall structure formation and allelopathy (Kroon and Williamson, 1999). Particularly in wheat, some phenolics have been postulated to have a role in dormancy regulation or susceptibility to preharvest sprouting (Weidner *et al*., 1999).

Wheat phenolics exist in several forms as free or simple soluble glycosides or esters with polysaccharides, proteins, or cell wall constituents. For instance, as phenolic acids; or in conjugation with lipids such as alkylresorcinols (Ross *et al*., 2003); or as polyphenolic compounds such as lignans (Peñalvo *et al*., 2005), lignins and flavanoids (Yun and McDonald, 1989). Phenolics are important determinants of wheat flour quality, primarily due to their role in pigmentation of flour and bread (McCallum and Walker, 1990), and additionally their influence on flavor, texture and color. Phenolics in a wheat grain also contribute to the nutritional and antioxidant properties of food (Kequan and Liangli, 2004; Espositio *et al*., 2005). In a wheat grain, phenolics are located in the outer bran layers. The specific location of phenolics has been used in bran fractionation to produce new functional food ingredients (Antoine *et al*., 2004). Ferulic acid is the main phenolic acid in wheat, present in the bran. From a nutritional perspective, ferulic acid possesses antioxidative activity and crosslinks with arabinoxylans to

produce insoluble dietary fiber (Peyron *et al*., 2001). Thus, phenolics contribute to the beneficial effects derived from consumption of wheat bran.

## 2.3 Starch: Food and industrial applications

Starch is the reserve energy source in plants, which is utilized during germination. Different plants have different starch storing organs, being seeds, fruits, tubers and roots. Humans acquire up to 80% of their daily calories from starch-based foods (Jobling, 2004). From the industrial perspective, starch serves as a source of bioethanol (Röper, 2002) being an inexpensive and renewable raw material (Jenkins and Donald, 1995; Nakamura, 2002). With the increase in understanding of starch structure and biosynthesis, and the advent of genetic modification, physico-chemical properties of starch can be genetically modified, and starches with novel functionalities can be synthesized in plants (Davis *et al*., 2003).

### 2.3.1 Current uses of starch

Starch has been extensively used in food processing, feed and industrial applications (Delcour *et al*., 2010). Primary uses of starch include production of food and beverages, manufacturing of adhesives, cosmetics, detergents, paper, and textiles (Ellis *et al*., 1998). Production of biodegradable packing material from starch is an alternate to petroleum-based products (Riaz, 1999). Biodegradable plastics including thin films (Lim *et al*., 1992) and molded plastic products (Albertsson and Karlsson, 1995) have also been manufactured from native starch granules. In addition, native wheat starch granules are also used to separate sheets of carbonless copy paper to prevent ink blotting (Nachtergaele and Van Nuffel, 1989; Maningat and Seib, 1997).

The food industry has used the biochemical properties of starch as thickeners for soups, sauces and dairy products, gum candies and other confectionary products, starch-based fat replacers to maintain the texture, flavor and mouth-feel of low- or no-fat products. (Maningat and Seib, 1997; Ellis *et al*., 1998). The property of starch to form visco-elastic pastes on heating in water has also been used in the food industry. Upon heating in water, amylopectin melts and starch granules swell, which eventually leads to disintegration of granule structure and solubilization of starch. Upon cooling, the disrupted chains aggregate, precipitate and form a gel.

This process is known as retrogradation, and starch at this stage is tolerant to changes in temperature and pH, thus can be processed under a variety of conditions (Jobling, 2004).

In addition to the food industry, there are many other industrial uses for starch (Davis *et al*., 2003), which include paper coating and adhesives for amylopectin-rich starches (Slattery *et al*., 2000), as joint compounds in construction industry (Maningat and Seib, 1997), and as fillers for biodegradable plastics from granular (Jacobsen and Fritz, 1996) and gelatinized starches (Verhoogt *et al*., 1995). Recent examples include the use of starch granules as delivery vehicles that protect pharmaceutically active proteins from digestion (Ali and AlArifi, 2009), as microencapsules for small molecules (Korus *et al*., 2003) and as biodegradable films (Rindlay-Westling *et al*., 2002; Weber *et al*., 2002).

### 2.3.2 Modified starch functionality

Starch is modified using chemical and molecular techniques to improve its functionality. Starch characteristics such as granule shape and size, amylose to amylopectin ratio, amylopectin chain length, degree of phosphorylation etc. play a significant role in altering starch behaviour and functionality (Santelia and Zeeman, 2011). Thus, starch structural and compositional parameters should be considered before modification for altered end-uses.

***Amylose: amylopectin ratio***

Amylose and amylopectin behave differently when heated-cooled in water, which leads to different gelatinization and re-crystallization properties. After the heating-cooling cycle, amylopectin forms viscous solutions stable at room temperature, while amylose retrogrades into a gel stable at temperatures greater than 60-70°C. Thus, reduced amylose concentration starches are desirable in processed foods, as they exhibit freeze-thaw stability (Hermansson and Svegmark, 1996). Amylose-free starch was produced by antisense down-regulation of starch synthases in potato which possessed exceptional freeze-thaw stability (Jobling *et al*., 2002). Waxy and non-waxy wheat and corn starches were studied previously, for the physicochemical characteristics of flours (Abdel-Aal *et al*., 2002). Waxy wheat flours showed high levels of water absorption, peak viscosity, gelatinization temperatures, greater degree of crystallinity, refrigeration and freeze-thaw stabilities; thus suggesting the importance of amylose-free flours in dough preparation and the baking industry. High-amylose starches are characterized by higher

gelatinization temperatures and lower peak viscosities (Blennow *et al*., 2001; Hofvander *et al*., 2004). High gelling strength and film-forming ability of these starches make them useful in the production of corrugated board, paper and adhesive products. High-amylose starch was produced by antisense inhibition of starch branching enzymes in potato (Schwall *et al*., 2000).

***Degree of phosphorylation***

High-phosphate starches have increased water-binding capacity, lower crystallinity, and low swelling temperature (Muhrbeck and Eliasson, 1991). These properties make them less likely to retrograde, improve viscosity and transparency, leading to freeze-thaw stability (Blennow *et al*., 2001). Covalently-bound phosphate provides an overall charge to the starch making it useful as surface coatings in the paper industry (Blennow *et al*., 2003). Potato tuber starch is highly phosphorylated, while cereal starches are essentially phosphate-free. Creating phosphorylated cereal starches could diversify their end-uses.

***Starch granule size***

Starch granule size varies with botanical source. Rice starch contains small granules of uniform size (~5 μm), while potato starch consists of larger granules up to ~100 μm in diameter. In wheat, barley, and rye, large lenticular and small spherical starch granules co-exist (Lindeboom *et al*., 2004). Starch granule size is a major determinant of starch to be used as filler in paper-making industry (Wang *et al*., 2003). Large and small starch granules confer different properties. For example, large granules have a higher swelling power and high viscosity; whereas small granules have lower gelatinization temperatures and give a smoother paste texture (Lindeboom *et al*., 2004). Furthermore, starch granule size is important in the brewing process. During filtration, small starch granules from barley do not get completely gelatinized in the mash and cause mechanical problems (Tillett and Bryce, 1993).

## 2.4 Starch granule: Composition and structure

In nature, starch granules occur in all shapes and sizes (spheres, ellipsoids, polygon, platelets and irregular tubules) with diameters ranging from around 0.1 to 200 μm depending on their botanical origin (Gallant *et al*., 1992; Jane *et al*., 1994; Hoover, 2001; Tester *et al*., 2004; Srichuwong *et al*., 2005). In plants like wheat and barley, one starch granule is produced individually per amyloplast. Such starch granules are called ‘simple’. However, in some plants

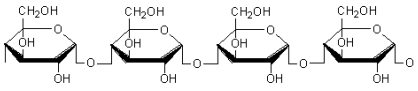
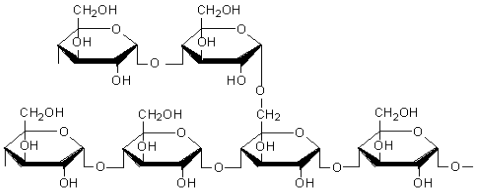
more than one granule is produced simultaneously in a single amyloplast making them difficult to separate. These examples of 'compound' starches are found in rice, oats and wrinkled peas.

### 2.4.1 Amylose and amylopectin

Starch is a glucan homo-polymer composed of amylose and amylopectin. Both these components differ in structure and properties. One-quarter of starch is amylose and three-quarters amylopectin; the former is essentially linear, while the latter is highly branched (Table 2.1). Glucose residues in a linear chain are linked via α-1,4 glucosidic linkages while α-1,6 glucosidic linkages form branches. The average number of chains in a single amylose molecule varies from 5 (rice and maize) to 21 chains (wheat) (Shibanuma *et al*., 1994). Short chains of amylose comprise small clusters along the long chains. The actual unit of branched amylose is not known, because the components cannot be separated from the linear portion.

Amylopectin shows variation with respect to the unit chain lengths and branching patterns. Individual chains can be allocated into different groups depending on their lengths and position within the starch granule (Hizukuri, 1985; 1986) (Figure 2.3). The glucose chain, bearing the sole reducing end, which within the starch granule is oriented towards the center or hilum of the granule, is called the C-chain. It consists of >36 glucose residues. The A- and B1-chains are the outermost chains which form double helices within a native starch granule. A- and B1-chain lengths vary from 6-12 and 13-24 respectively, depending on genetic origin (Hizukuri, 1985; Li *et al*., 2001; Franco *et al*., 2002). Other longer B-chains can span and support other clusters and chains. Depending on the chain length and correspondingly the number of (radial) clusters traversed within a native starch granule, B-chains are referred to as B1 – B3 (one to three clusters). Typical chain lengths for A-, B1-, B2-, and B3-chains for different starches (after debranching with isoamylase) are 6-12, 12-24, 40-50, and 60-80 respectively (Hizukuri, 1986; BelloPérez *et al*., 1996; Mua and Jackson, 1997). The degree of branching, entanglements between amylose and amylopectin, along with the presence of phospholipids and lipids, have profound effects on physical and biological properties of starch granules.

Table 2.1 Characteristics of amylose and amylopectin

| | **Amylose** | **Amylopectin** |
|---|---|---|
| Structure |  |  |
| Linkages | Predominantly linear, α-1,4 glucosidic linkages | Highly branched, α-1,4 and α-1,6 glucosidic linkages |
| Branching frequency | Few branches (1 per 1000 residues). Large molecules have up to 10 or more (Hizukuri, 1986) | 4-5% branching<br>1 per 20-25 residues |
| Percent occupancy | ~15-35% | ~75% |
| Molecular mass | $10^5$-$10^6$ Da | $10^7$-$10^9$ Da |
| Degree of polymerization | 830-1570 in different wheat varieties (Shibanuma *et al*., 1994) | $10^5$-$10^7$ |
| Chain length | Longer chains with hundreds to thousands of residues, with small amounts (3.2-7.6% weight basis) of very short chains | Chains relatively short with a broad distribution profile |

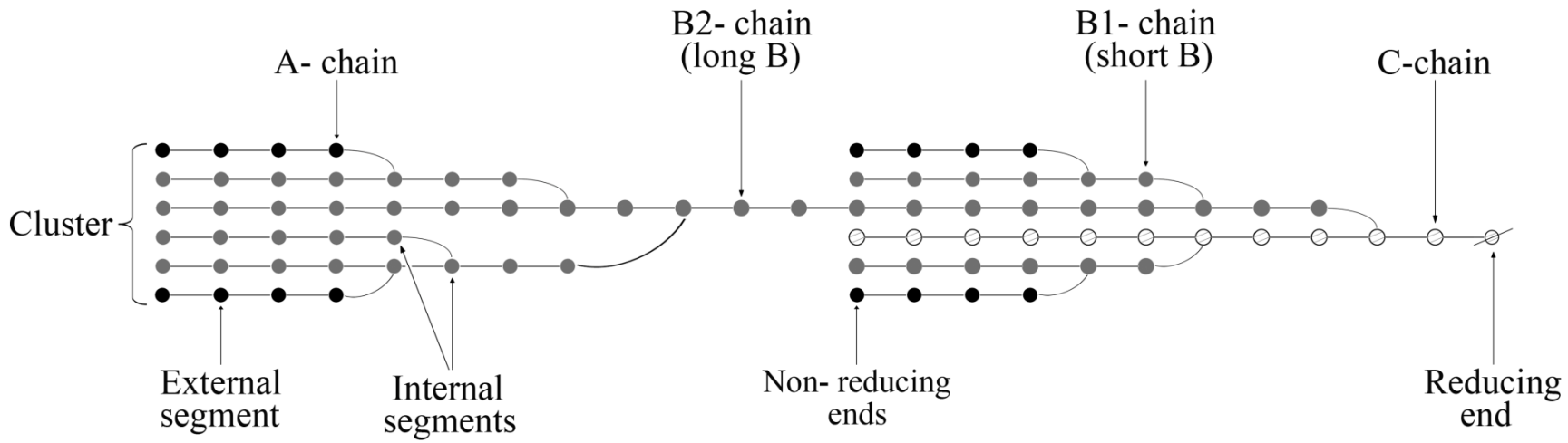



Figure 2.3 Amylopectin chain length distribution

Circles indicate glucosyl residues in C-chain (white hollow), B-chain (grey) and A-chain (black). Horizontal lines represent α-1,4 glucosidic linear linkages and bent lines indicate α-1,6 glucosidic branch linkages. Redrawn from Pérez and Bertoft, 2010.

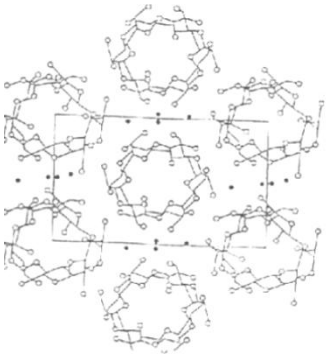
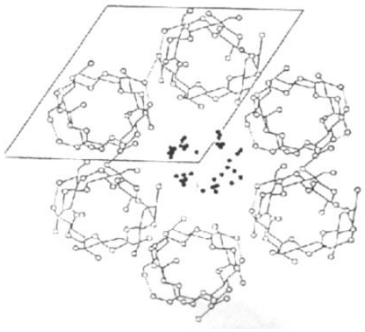
### 2.4.2 A- and B-type Crystallinity

Outer chains of amylopectin intertwine with each other forming double helices, which when present in close proximity tend to form three-dimensional geometric patterns, hence crystallinity. According to the rules of crystallography, such an array of atoms, molecules or group of molecules, will interact with electromagnetic waves of short wavelength (X-ray) to give rise to a characteristic diffraction pattern (Tester *et al*., 2004). Native starch granules differ in their morphology and crystallinity, hence exhibit different X-ray diffraction patterns based on which they are classified into A-type and B-type crystalline starches (Hung *et al*., 2008) (Table 2.2). The amount of loss of entropy upon crystallization is different for chains of different lengths (Nishiyama *et al*., 2010). Hence, amylose chain length plays a role in the type of crystalline form. The double helices in both A- and B-type crystalline forms are left-handed, as it is energetically more preferable (Imberty *et al.*, 1988a; 1988b; Buléon *et al.*, 1998). As there is a difference in the repeating units, a relative translational shift of 0.5 nm along the orientation of the chains occurs, which allows a very close nesting of the 'crests' and 'troughs' of the paired double helices (Pérez *et al*., 1990). In addition, a third C-type crystallinity also exists, which is a mixture of A- and B-types of crystallinity (Wang *et al*., 2008). Legume starches such as pea contain both A- and B-type crystalline structure, with the A-type structure in the periphery and B-type in the centre of the starch granule (Gernat *et al*., 1990). Another crystalline V-form characteristic of amylose complexed with fatty acids and monoglycerides, which appears upon gelatinization of starch, is rarely detected in native starches (Buléon *et al*., 1998).

### 2.4.3 A-, B- and C-type Starch granules

Starch granules are synthesized in the amyloplast of the wheat endosperm. Previously, a bimodal type of starch granule size distribution was reported. But more recently, a trimodal rather than bimodal distribution has been reported (Bechtel *et al*., 1990; Raeker *et al*., 1998). Granules of different shapes and sizes are developed in the endosperm during different periods of grain development. In the initial grain development period, A-type starch granules are formed, followed by B- and C-type starch granules in the later stages of development (Rahman *et al*., 2000). A-type starch granules initiate at 4-5 days post anthesis, continue to increase in number

Table 2.2 Comparison of A- and B-type crystalline starches

| **A- Type Crystallinity** | **B- Type Crystallinity** |
|---|---|
|  |  |
| Cereals like corn (Abd Allah *et al*., 1987), wheat (Hung *et al*., 2008), barley (Song and Jane, 2000), rice (Kubo *et al*., 2008) | Root and tuber starches of potato (Kim and Lee, 2002), tapioca (Butrim *et al*., 2009), taro (Hoover, 2001) |
| Caused by amylopectin with short average lateral branch chains (DP 10-12) (Buléon *et al*., 1998) | Derived from amylopectin with long side chains containing distant branching points (DP > 12) (Butrim *et al*., 2009) |
| Double-helices packed in an anti-parallel hexagonal mode. Central channel occupied by another double helix, making the packing more compact. | Double-helices packed in an anti-parallel hexagonal mode. Central channel surrounded by 6 double helices is filled with water. |
| 8 $H_2O$ / unit cell (Wu and Sarko, 1978a) | 36 $H_2O$ / unit cell (Wu and Sarko, 1978b) |
| Repeating unit is a maltotriosyl unit (Imberty *et al*., 1988a) | Repeating unit is a maltosyl unit (Imberty *et al*., 1988b) |
| Form under warmer, drier conditions | Form under cool, wet conditions |

until 7 days (Briarty *et al*., 1979), where after their number remains constant but they continue to grow in size (10-35 μm) throughout the grain filling period (Dengate and Meredith, 1984). B-type starch granules on the other hand, are initiated at 12-14 days post anthesis (Parker, 1985; Bechtel *et al*., 1993) and they increase in number and size (less than 10 μm) until grain maturity (Morrison, 1989). The third class of granules, C-type starch granules, is initiated at 21 days post anthesis. Although the number of A-type starch granules is less than B-type starch granules, but being larger in size they account for 70-80% of the starch mass (Stark and Lynn, 1992). B-type starch granules constitute ~95% of the granule numbers in the endosperm (Morell *et al*., 1995). Each amyloplast produces one A-type starch granule. Formation of B-type starch granules however, initiates in A-type amyloplasts (amyloplasts containing one A-type starch granule). Maintenance of a constant number of A-type starch granules throughout development is because either there is turnover of A-type starch granules, or that few B-type starch granules develop beyond 10 μm in diameter (Bechtel *et al*., 1990; Morell *et al*., 1995).

A-type and B-type starch granules have different formation patterns. A-type starch granules develop by equatorial plate formation (Evers, 1971). Initially, these are spherical granules of 0.5-1 μm diameter, which grow radially to 2-4 μm. Then a bulbous protrusion develops at one end, which develops into an apposition plate, extends around the granule and eventually encircles it. The equatorial plate extends till the maximum granule diameter is achieved. Active deposition of glucan molecules then takes place over the equatorial plate, giving the characteristic lenticular shape to the mature wheat A-type starch granule. This deposition proceeds in a circadian manner, thus producing alternating layers in a starch granule (Rahman *et al*., 2000). B-type starch granules are produced in the lateral evaginations of the amyloplast membrane. Multiple small B-type starch granules are present in a single evagination. Unlike A-type, B-type starch granules do not undergo the process of equatorial plate formation and hence remain spherical or orthorhombic (Parker, 1985).

Differences in the orientation of amylopectin molecules in A- and B-type starch granules were reported (Ao and Jane, 2007). In a disc-shaped granule such as the A-type starch granule of wheat, triticale and barley starch, the amylopectin molecules are oriented perpendicular to the equatorial plane of the disc-shaped granule and are aligned parallel. Therefore, they would consist of more B2-glucan chains and few short A- and B1-glucan chains. On the other hand, B-

type starch granules, which are spherical, consist of shorter glucan chains (A and B1) and few long B2-glucan chains.

Starch granules of different shapes and sizes exhibit different physicochemical properties, hence determining their end-uses. For example in wheat, lenticular shaped large A-type starch granules have been used for carbonless copy paper, while spherical small B-type starch granules are good plastic film fillers.

### 2.4.4 Amylopectin structure

Amylopectin exhibits a high degree of architectural specificity despite containing only one type of monomeric unit (i.e. the glucosyl group) connected through just two types of linkages (Myers *et al*., 2000). Amylopectin architecture can be understood at different scales (Figure 2.4).

*0.1 – 1.0 nm scale*: Structure is described by individual chains; their lengths and branch locations. It has been reported that the frequency of chains of a specific length is generally conserved in amylopectin from different plants (Morrell *et al*., 1988). Enzymatic treatments of starch have revealed that the branches occur in clusters (Myers *et al*., 2000).

*10 nm scale*: This level of measurement describes alternating crystalline and amorphous lamellae. Crystalline lamellae are resistant to acid hydrolysis, while amorphous lamellae are susceptible. Crystalline lamellae consist of tightly associated double-helices containing A-glucan chains and unsubstituted B-glucan chains; while amorphous lamellae contain frequent branch linkages making it less densely packed. The 9-10 nm thickness of this repeating unit is conserved in the plant kingdom (Jenkins *et al*., 1993).

Amylopectin chain length clusters are 10 nm wide discrete units within the crystalline lamellae (Gallant *et al*., 1997). Regions of relatively lower branch frequency (DP 11-15) form double-helices in the amylopectin side chain clusters. The short chains which do not form double helices introduce structural defects in the crystalline lamellae and thereby interfere with the organization of crystals (Fredriksson *et al*., 1998; Nakamura *et al*., 2002; Inouchi *et al*., 2005; Genkina *et al*., 2007; Kong *et al*., 2008; Noda *et al*., 2009; Singh *et al*., 2009; Wang *et al*., 2010). Formation of clusters at this level provides a foundation for organization of chains at a higher level. Specific chain length distributions and arrangements may be required for side chain clusters to be energetically favorable (Myers *et al*., 2000).

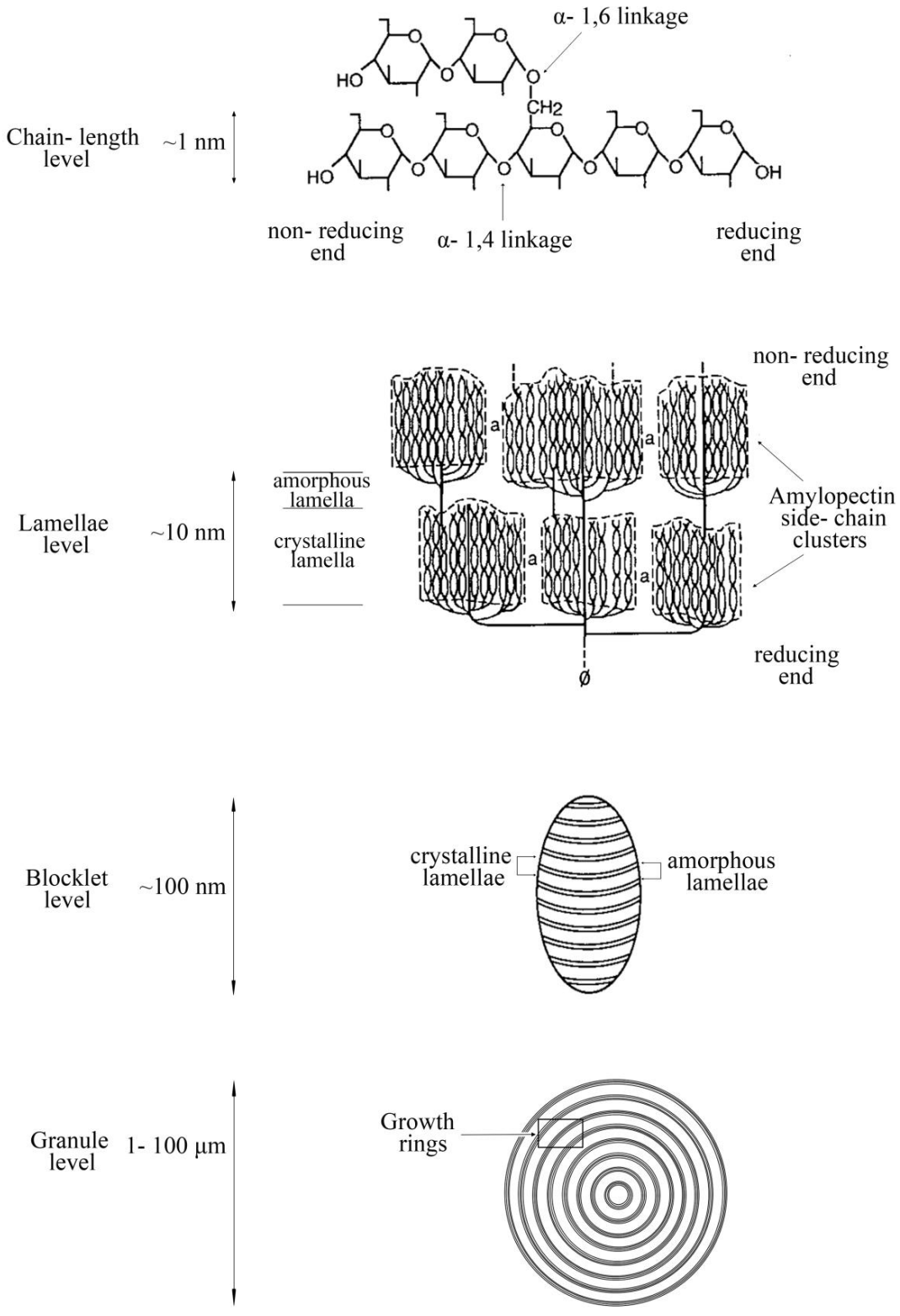



Figure 2.4 Amylopectin architecture at different scales

Adapted from Myers *et al*., 2000; James *et al*., 2003.

The amorphous zones have long been considered to be single helical amylose, based on nuclear magnetic resonance (NMR) studies (Gidley and Bociek, 1988). Amylose molecules are dispersed among the amylopectin molecules, present in the amorphous regions (Jane *et al*., 1992). It was reasoned that the amylose is synthesized in spaces inside the matrix, rather than at the surface in the same location as amylopectin synthesis. Hence it does not form part of the crystalline matrix (Smith, 2001).

*100 nm scale*: The next structural level is the 'Blocklet' (Gallant *et al*., 1997). Crystalline and amorphous lamellae, surrounded by non-crystalline regions comprise one blocklet. These discrete elongated structures were observed by transmission electron microscope. Areas between blocklets are about 10-20 nm wide. One blocklet is approximately 20-500 nm in diameter, which would vary in shape and size depending on starch botanical type and their location in the granule (Gallant *et al.*, 1997). Size of a blocklet varies with the crystallinity as well. For example, in potato (B-type crystallinity) a typical blocklet is 400-500 nm in diameter; as compared to wheat (A-type crystallinity) where its diameter is 25-100 nm. In wheat starches, larger (100 nm) blocklets are found in the hard semi-crystalline rings of the granule than in the softer amorphous rings, where blocklet size is around 25 nm (Perez and Bertoft, 2010).

One blocklet hosts a single amylopectin macromolecule, and a larger blocklet would consist of several amylopectin molecules (Blennow *et al*., 2001; Takeda *et al*., 2003). A blocklet is constructed by crystalline and amorphous lamellae, which are formed by the clusters of amylopectin molecules. The reducing terminal of amylopectin in a blocklet would be towards the hilum of the granule. Blocklets are believed to be aligned relative to each other in various ways, rather than having a common orientation (Myers *et al*., 2000).

*120-150 nm scale*: This is the 'growth ring' structural level, which has been observed as characteristic alternating rings in a starch granule. Growth rings are composed of blocklets, which could vary in size and/or packing geometry in different rings (Myers *et al*., 2000). These internal semi-crystalline growth rings are differentially sensitive to chemical and enzymatic attack, with dense layers being more resistant. These may be regions of close packing of branches within a branch cluster of parallel amylopectin molecules (Martin and Smith, 1995).

The formation of growth rings occurs because the structure of starch laid down at night is different from that laid down during the day (Smith, 2001). It was reported that in wheat, growth

rings follow a clear diurnal pattern which is lost in plants grown under continuous light (Buttrose, 1962; French, 1984). However the presence of growth rings was found in potato tubers, which develop under constant conditions of light and temperature (Buttrose, 1960; 1962). Therefore, several factors both biological and physical might affect the formation of growth rings. The degree of interaction between these factors may vary between starch-storing organs in different plant systems (Smith, 2001).

***Cluster structure of amylopectin***

Structural studies were performed by removing glucan branches of starch by acid treatment, which revealed that they exist in groups as clusters (Watanabe and French, 1980; Biliaderis *et al*., 1981). The size of clusters and density of branching differs with crystallinity. A-type crystalline starch has larger clusters with higher branching density than B-type crystalline starch (Bertoft and Koch, 2000; Gerard *et al*., 2000; Kong *et al*., 2009).

The most widely accepted cluster structure model suggests the participation of A- and B-type glucan chains (Hizukuri, 1986). According to this model, a cluster is built from short A- and B1-chains. These clusters are interconnected by long B-chains, which intersperse crystalline as well as amorphous regions. Since amorphous chain segments can be found in several directions, therefore the internal chain length of long B-chains does not necessarily correlate with the thickness of an amorphous lamella. This cluster model has been tested by cleaving long B-chains using α-amylase, suggesting their role in interconnection of clusters. These studies have been performed in different plant systems such as maize, rice, amaranth, cassava and potato (Bertoft, 1991; Bertoft and Koch, 2000; Bertoft, 2007; Kong *et al*., 2009; Laohaphatanaleart *et al*., 2010).

Within clusters, the branches exist in groups, which are the building blocks of the starch granule. The number of building blocks that make up a cluster vary with the size of the blocks and clusters. The degree of polymerization of building blocks ranges from 5 to 40 glucan molecules. Within a building block, the internal chain length is short (2-3 glucan molecules), therefore the distance between branches in this densely packed area is ~1-4 glucosyl residues. Internal segments exist between clusters, between building blocks inside clusters and inside building blocks (Bertoft, 2007). Three models for cluster interconnections have been proposed (Bertoft, 2004); (i) Two-directional backbone model, in which the direction of clusters is perpendicular to the direction of the backbone, hence affecting the direction of crystalline and

amorphous lamellae; (ii) some long B2-chains participate in both cluster interconnections and in the amorphous backbone, while some along with B3-chains, are present only in the backbone. This was suggested for potato; and (iii) the cluster itself forms a part of the backbone through its inter-block segments. It agrees with the liquid crystalline model of amylopectin (Waigh *et al*., 2000). In either case, if chains connected to the backbone are very small, they might introduce structural defects in the crystalline lamella rather than promoting crystallinity (Koroteeva *et al*., 2007; Kozlov *et al*., 2007a; 2007b).

The location of the amylopectin branch points has been proposed to be in the amorphous regions between crystallites. Under low energy arrangements, at branch points, side chains fold back onto the carrying chain, in a parallel fashion, thereby producing dense 3D structure (Pérez and Bertoft, 2010). In addition two double helices could be connected at a branch point, with minimal distortion. This suggests that the branch points do not induce defects in the double helical structure, but instead serve to initiate the crystalline arrangement.

### 2.4.5 Location of amylose in starch granules

It is unclear how amylose interacts with amylopectin in a starch granule. Three hypotheses for the location of amylose in the granule have been proposed. The first hypothesis suggests that amylose is laid down tangentially to the radial orientation of amylopectin, hence forming the amorphous region. This would result in minimal amylose-amylopectin interactions (Gidley, 1992). The second and third hypotheses suggest that amylose is laid down radially to exist either in bundles (Nikuni, 1969; Zobel, 1992) or as individual chains (Jane and Shen, 1993; Kasemsuwan and Jane, 1994), respectively. This would result in randomly interspersed amylose within amorphous and crystalline regions. According to these hypotheses, amylose chains cross-link with amylopectin chains. X-ray scattering experiments showed that starches with increasing amylose content caused changes in the ratio of amorphous to crystalline lamellae within the unit amylopectin clusters (Jenkins and Donald, 1995). This could be because of amylose disrupting the packing of amylopectin double helices within the crystalline lamellae either by crystallizing with amylopectin and pulling the amylopectin chains out of the crystalline structure or, by being oriented transverse to amylopectin stacks. Therefore, this experiment agrees with the third hypothesis of individual amylose chains being randomly distributed amongst radial amylopectin chains (Pérez and Bertoft, 2010).

Although most of the amylose chains exist as single helices, however, a small portion of amylose exists complexed with lipid. There are two beliefs regarding the location of lipid complexed amylose in the granule. Complexes between amylose and lipid were reported at the core of granules (Morrison, 1989). However, recent reports indicate the enrichment of amylose and lipid at the periphery of the granule (Boyer *et al*., 1976; Shannon *et al*., 1984; Morrison and Gadan, 1987). In addition to lipid complexed with amylose, free lipid fraction also exists within the granule (Blanshard, 1987).

Another interesting fact is that the amylose found near the surface of the granule has a smaller chain length than amylose located nearer the centre of the granule (Boyer *et al*., 1976).

## 2.5 Starch granule biosynthesis

A summary of the enzymes involved in the starch biosynthesis pathway is shown in Figure 2.5.

### 2.5.1 Formation of hexose phosphates by photosynthesis

In the chloroplast of photosynthetic organs such as leaves, sunlight is harvested by the photosystems in the thylakoid membrane and result in the formation of reducing equivalents and ATP. ATP thus synthesized, produces triose phosphates through carbon reactions which take place in the chloroplast stroma. Triose phosphates either remain in the plastid, or, are transported into the cytosol via Pi translocators. In the plastid, they are used for the production of hexose phosphates viz., fructose-6-phosphate (F6P), glucose-6-phosphate (G6P) and glucose-1-phosphate (G1P). G1P is utilized for the formation of ADP-glucose by ADP-glucose pyrophosphorylase, which is the first reaction for starch synthesis. Triose phosphates which are transported to the cytosol are converted to sucrose via hexose phosphates. Sucrose is converted to UDP glucose and fructose by sucrose synthase (SuSy). UDP glucose is subsequently converted to G1P by UDP-glucose pyrophosphorylase, which is converted to G6P by phosphoglucomutase. UDP-glucose pyrophosphorylase has been shown to be very active in wheat endosperm (Turner, 1969; Duffus, 1992). G6P is transported to the plastid via specific glucose-6-phosphate/ phosphate transporter and the ATP/ADP transporter present in the plastid envelope (Neuhaus and Wagner, 2000). Previous studies have shown that mostly hexose phosphates, not triose phosphates, are translocated into the plastid for starch synthesis (Keeling *et al*., 1988; Tyson and Rees, 1988; Neuhaus *et al*., 1993). Inside the plastid, G6P is converted to

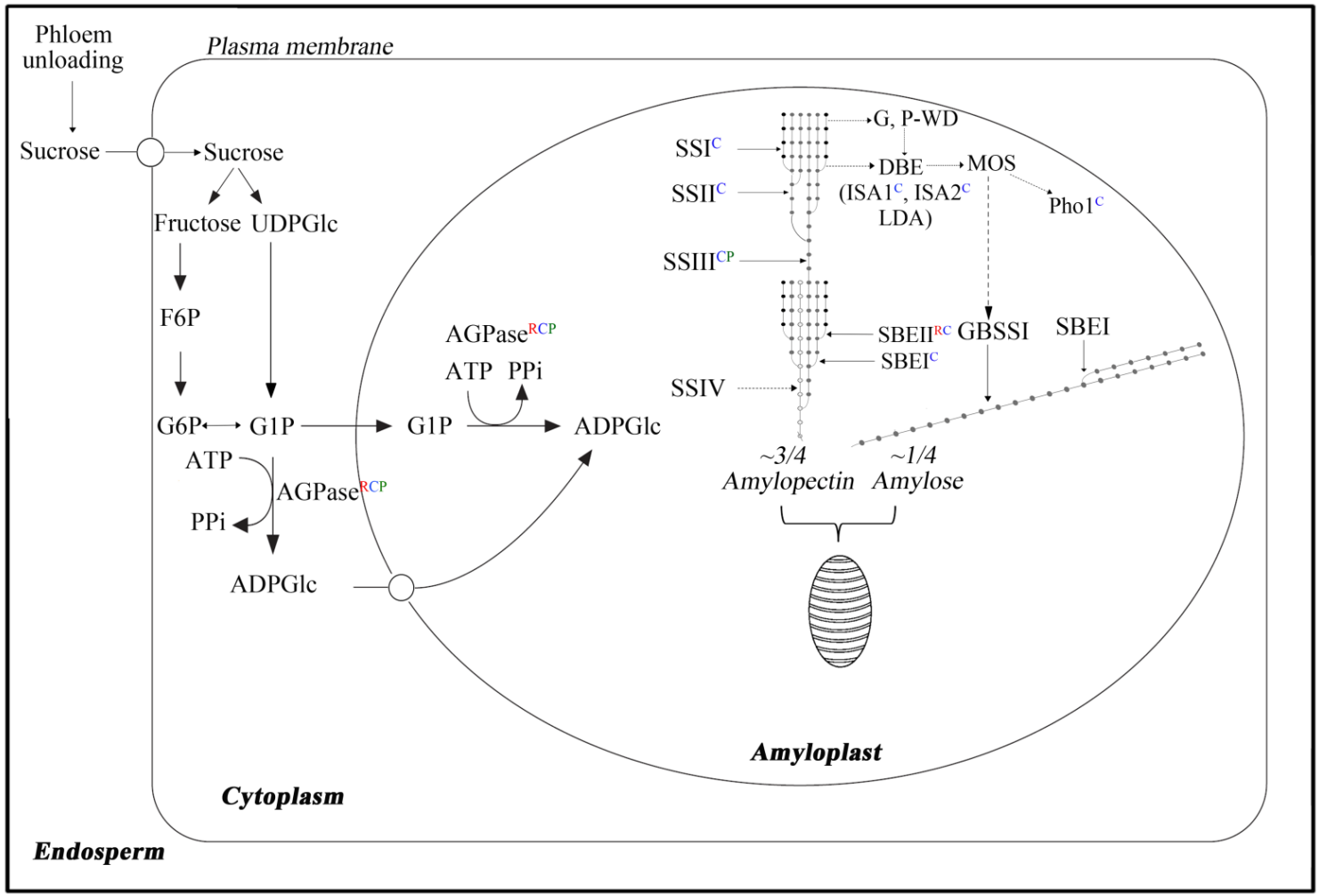



Figure 2.5 Schematic representation of starch biosynthesis in wheat endosperm

Abbreviations used are: UDPGlc: UDP glucose, F6P: Fructose-6-phosphate, G6P: Glucose-6-phosphate, G1P: Glucose-1-phosphate, MOS: Maltooligosaccharides. R – Redox regulated, C – Complex formation, P – Phosphorylation regulated. The diagram is based on reviews by Rahman *et al*., 2000; Tetlow, 2011.

G1P by phosphoglucomutase, hence providing substrate for starch synthesis. Starch produced in leaves is called transient starch, which is degraded in the following night to provide carbon for respiration and other plant metabolic activities. In non-photosynthetic, starch storing organs such as wheat seeds, starch is synthesized from sucrose which is imported into the endosperm of the grain from leaves. During the development of the grain, sucrose unloads from the phloem, passes through chalaza to nucellus, from where it is transported to the developing endosperm via transfer cells of the aleurone layer (Thorne, 1985; Emes *et al*., 2003). After endosperm proper is formed, sucrose is unloaded from the phloem into the vascular bundles situated embedded in a furrow running along the length of the kernel, and further passes through various cell layers to reach the inside of the grain. Once inside the endosperm cell, sucrose is catabolised by SuSy to form G6P, which when transported to amyloplast, acts as the substrate for starch synthesis. Endospermic SuSy plays an important role in starch synthesis, with increased activity during dark periods when there is a high rate of sucrose unloading (Dale and Housley, 1986).

### 2.5.2 Formation of ADP-glucose by ADP-glucose pyrophosphorylase

ADP-glucose pyrophosphorylase (AGPase) is the first and rate limiting enzyme committed to transient and storage starch synthesis. It catalyzes the synthesis of ADP-glucose and pyrophosphate from ATP and G1P (Smith *et al*., 1997). Glucose from ADP-glucose is utilized by other enzymes for starch biosynthesis in plants and glycogen synthesis in bacteria. Until recently, it was believed that AGPase was located exclusively in plastids. However, later studies showed the existence of two distinct isoforms of AGPase in cereals viz. maize (Denyer *et al*., 1996b), barley (Thorbjørnsen *et al*., 1996), rice (Sikka *et al*., 2001) and wheat (Tetlow *et al*., 2003). These are known as plastidial and cytosolic isoforms of AGPase. The cytosolic form of AGPase accounts for 65-95% of the total AGPase activity in developing cereal endosperms, implying that imported ADP-glucose is the major substrate for storage starch biosynthesis. The reason is that in cereal endosperm, the cytosolic pathway may offer a selective advantage by drawing upon the relatively abundant cytosolic pool of ATP rather than the more limited pool of ATP in the plastid.

Plant AGPase is a heterotetrameric enzyme, composed of two subunit types, the ‘large’ (55-60 kDa) and ‘small’ (50-55 kDa) subunits, in a presumably $\alpha_2\beta_2$ configuration (Morell *et al*., 1987; Preiss, 1991). cDNA clones encoding both subunit types have been isolated from several

plant species (Olive *et al*., 1989; Bae *et al*., 1990; Bhave *et al*., 1990; Villand *et al*., 1992a; 1992b; Weber *et al*., 1995), which show considerable homology, indicating they may have arisen from a gene duplication event (Smith-White and Preiss, 1992). Immunological cross-reactivities and sequence comparisons suggest that small subunit structure is highly conserved among plant groups and between photosynthetic and non-photosynthetic tissues, whereas the large subunits are more divergent (Smith-White and Preiss, 1992). In hexaploid wheat, cDNAs encoding each of the endosperm and leaf AGPase subunits have been cloned (Olive *et al*., 1989; Ainsworth *et al*., 1993). The genes for the small subunit are organized as single-copy loci on chromosomes 7A, 7B and 7D (Ainsworth *et al*., 1993).

Different species possess different number and different expression patterns of genes encoding each subunit. For example, in potato, three transcripts encoding the large subunit, all of which are expressed in tubers, while only two are expressed in leaves; and one transcript encoding small subunit have been reported (Cognata *et al*., 1995). Expression of each of the genes encoding the subunits is differently regulated by developmental and metabolic signals. In maize, there are two genes encoding the large and three encoding the small subunits. Transcripts for one of the small subunit genes (*Brittle2*) are detected only in endosperm, but transcripts of a second gene (*Agp1*) are present in the embryo and also at low levels in the endosperm; expression of the third gene has been detected only in the leaf (Giroux and Hannah, 1994). In barley there are two genes encoding the large subunit – one expressed only in the endosperm and the other primarily in the leaves; but only one encoding the small subunit. However, the gene encoding small subunit produces two transcripts, with one expressed only in endosperm, and the other majorly in leaves and minorly in endosperm. These two transcripts vary only at their 5' ends and are identical for the rest 90% of their sequence (Thorbjørnsen *et al*., 1996).

Both, large and small subunits can sustain enzymatic activity in the absence of the other subunit although the turnover rate of such enzymes is greatly diminished (Preiss *et al*., 1990; Iglesias *et al*., 1993). Maize *Brittle2* mutant (which affects the small subunit in endosperm) and the maize *Shrunken2* mutant (which affects the large subunit in endosperm) abolish 90-95% of the AGPase activity in the endosperm, giving up to 75% less starch (Tsai and Nelson, 1966; Bae *et al*., 1990; Bhave *et al*., 1990). In pea, mutation at the *Rugosus-b* locus affects a gene encoding the large subunit and results in a 90% or greater reduction of AGPase activity and a concomitant drop of ~50% starch accumulation (Hylton and Smith, 1992). These results show that each

subunit contains those residues absolutely required for catalysis and that the presence of the companion unit promotes a much favorable conformation.

In addition to affecting the amount of starch produced, AGPase appears to influence starch composition. This is suggested by the fact that the *Rugosus-b* mutation of large subunit in pea not only reduces the total amount of starch produced but also alters its quality by increasing the proportion of amylopectin (Kooistra, 1962). A possible explanation states that reduced levels of ADP-glucose may have particularly extreme effects inside the starch granule, where availability is dependent on diffusion through the crystalline matrix. The reduced ADP-glucose availability could have a relatively greater effect on the amylose-specific GBSSI, which is located exclusively inside the granule, than on the SSs in the soluble phase, which synthesize amylopectin (Martin and Smith, 1995).

Most AGPases are subject to allosteric regulation by 3-phosphoglycerate (3-PGA, activator) and inorganic phosphate (Pi, inhibitor). Since AGPase is the rate limiting enzyme, its allosteric properties are important determinants of the rate of starch synthesis. In a study in *Chlamydomonas*, mutations in a gene (*STA1*) encoding one subunit of AGPase, reduce the sensitivity of AGPase to activation by 3-PGA, and reduces the rate of starch synthesis by 95%. However, the activity of AGPase is not altered in the absence of the activator (Ball *et al*., 1991; Koornhuyse *et al*., 1996), suggesting that alteration in an allosteric property can itself regulate the rate of starch synthesis, without affecting the total catalytic activity. Additionally, a high starch yielding line of maize has been isolated, where mutation was induced via transposon in a gene encoding the major large subunit of AGPase (*Shrunken2*). Endospermic AGPase of this variant is less sensitive to inhibition by Pi, along with reducing the total AGPase activity (Giroux *et al*., 1996).

Evidence that allosteric regulation is important for regulation of starch synthesis in cereals comes from studies of transgenic wheat containing maize AGPase genes that contain point mutations that confer stability to subunit interactions and reduce Pi inhibition (Smidansky *et al*., 2002). Analysis of a small number of these transgenic wheat plants showed they expressed a more stable and enzymatically active AGPase that, in some cases, was associated with increased seed weight and total biomass. However, there is also evidence that the regulation of AGPase in

cereal endosperm is less important than in other organs such as leaves (Kleczkowski, 1993a; 1993b; Gomez-Casati and Iglesias, 2002).

Pyridoxal phosphate analysis, which can be used to identify the site of 3-PGA binding, has shown that a conserved lysine residue at the C-terminus of the small subunit and the equivalent lysine residue in the large subunit binds this 3-PGA analog (Morell *et al*., 1988; Preiss, 1993). A second lysine residue in the large subunit from spinach leaf also binds pyridoxal phosphate; this residue may respond to Pi regulation (Preiss, 1993). However, this second lysine residue is not conserved within the large subunits from all sources. Most notably, in the large subunits expressed in wheat and barley endosperm it is replaced by methionine, although in the large subunit expressed in wheat leaves it is a lysine residue. This suggests that the degree of regulation exercised by 3-PGA and Pi could vary with the isoform of the large subunit expressed in different parts of the plant.

AGPase activity has been demonstrated to be affected by the redox status of the plastid stroma (Cho *et al*., 1999; Hendriks *et al*., 2003; Kolbe *et al*., 2005; Lunn *et al*., 2006; Sokolov *et al*., 2006). Regulation by redox affects the small subunits which, under oxidizing conditions, become covalently linked via an intermolecular disulfide bridge, thus forming a stable dimer within the heterotetramer (Fu *et al*., 1998; Tiessen *et al*., 2002; Hendriks *et al*., 2003). Dimerization leads to reduced AGPase activity and altered kinetic properties, by decreasing the enzyme's sensitivity to activation by 3-PGA and increasing its km for ATP (Tiessen *et al*., 2002; Hendriks *et al*., 2003). Monomerization of small subunits occurs under reducing conditions, making the enzyme active and sensitive to allosteric regulation. Redox regulation in chloroplasts resulted in increased AGPase activity during the day, hence a higher rate of starch synthesis; and reduced AGPase activity during the night. It is unclear, though, whether redox plays any role in determining the rate of starch synthesis in amyloplasts under normal growing conditions. It has been suggested that redox activation was mediated by thioredoxin, which was itself reduced through photosynthesis, or by NADP-thioredoxin reductase (Ballicora *et al*., 2000).

### 2.5.3 Glucan chain elongation by starch synthases

A major unresolved question of starch biosynthesis is the priming of polysaccharide synthesis. Because glycogen and amylopectin belong to the same family of storage

polysaccharides, it is suspected that synthesis of these molecules might be primed by a common mechanism which adds glucose units to the non-reducing end of the glucan chain. The first hypothesis for polyglucan priming, therefore, is that a protein with similar function in yeast and animal cells, auto-glycosylating glycogenin, the initiator of glycogen biosynthesis, is involved in the priming reaction (Cheng *et al*., 1995; Jenkins and Donald, 1995). However, later experiments showed that bacterial glycogen synthase and purified recombinant maize endosperm soluble starch synthase are capable of priming glucan synthesis in the presence of 0.5 M citrate (Imparl-Radosevitch *et al*., 1998). It is therefore quite possible that both starch and bacterial glycogen granule priming do not require the auto-glycosylating glycogenin like proteins. A second hypothesis excludes the need of a primer for initiation of glucan polymerization and suggests addition of glucose units to the reducing end of the glucan chain (Mukerjea and Robyt, 2005).

Glucan chain elongation is brought about by starch synthases, which add glucose residues to the non-reducing end of the growing glucan chain. Two major classes of starch synthases viz. 'granule-bound' and 'soluble' are known, with each existing in multiple isoforms. The major granule-bound starch synthase isoform in wheat endosperm is called GBSSI or 'Waxy' protein, which is primarily involved in amylose synthesis. It is encoded by 'waxy' loci located on wheat chromosomes 7AS, 4AL (translocated from 7BS) and 7DS (Demeke *et al*., 2000). Four isoforms, SSI, SSIIa, SSIIb and SSIII are present in soluble form and are involved in amylopectin synthesis. Multiple starch synthase isoforms are defined based on primary amino acid sequence relationships (Cao *et al*., 1999; Li *et al*., 1999). The C-terminal region of the coding sequence of the four soluble SSs contains several conserved regions including three short spans of amino acid domains I, II and III (Van der Leij *et al*., 1991), which are also present in glycogen synthase. Domain I contains the consensus sequence KXGGL which is the ADP-glucose binding site of the enzyme (Furukawa *et al*., 1993). Domains II and III lie closer to the C- terminus. The highest homology between the different starch synthase isoforms occurs from KXGGL through to the C-terminus. In addition to the C-terminal catalytic domain, soluble starch synthases contain a highly diverged 'flexible arm' at the N- terminus (Martin and Smith, 1995; Knight *et al*., 1998), which is absent in GBSSI.

***Granule-bound starch synthases***

GBSSI (58-60 kDa) is located inside and on the periphery of starch granules. The availability of waxy mutants from a number of species (wheat, barley, maize, rice and potato) demonstrates that granule-bound starch synthase is the enzyme responsible for the synthesis of the amylose fraction of starch (Shure *et al*., 1983; Preiss, 1991; Sivak *et al*., 1993). However, in addition to GBSSI, other factors or mechanisms might also be present for regulation of amylose synthesis. Because Km of GBSSI for ADP-glucose is five to ten folds higher than that of the soluble starch synthases, amylose synthesis is particularly sensitive to substantial decreases in the supply of ADP-glucose substrate. Hence, regulation of AGPase exerts some control on the final amylose content within the granule. In addition, amylose synthesis in chloroplasts of leaf cells compared with that of amyloplasts in sink tissues is somewhat more restricted because the chloroplast is often actively engaged in the export of photosynthate.

Different mechanisms for amylose synthesis have been proposed. *In vitro* studies concluded that malto-oligosaccharides diffuse into the granule matrix, and are used as primers by GBSSI for amylose synthesis (Leloir *et al*., 1961; Recondo and Leloir, 1961; Denyer *et al*., 1996a; Denyer *et al*., 2001). Other *in vitro* experiments suggest that given extended incubation times, GBSSI uses amylopectin as primer for amylose synthesis (Dauvillée *et al*., 1999; Denyer *et al*., 1999a; 1999b). It is hypothesized that elongated amylopectin chains are confined in the semi-crystalline granule structure and have no opportunity to entangle with other chains for branching reactions to take place. Consequently, the chains remain essentially linear, ultimately forming amylose (Denyer *et al*., 1996a; Jane *et al*., 2010).

In addition to amylose synthesis, GBSSI has been shown to be involved in amylopectin synthesis as well. Earlier studies have shown a difference in the amylopectin structure between waxy and non-waxy lines in *Chlamydomonas*, cereals and potatoes (Delrue *et al*., 1992; Maddelein *et al*., 1994; Flipse *et al*., 1996). Specifically, GBSSI deficient mutants lack long chain fraction of amylopectin in *Chlamydomonas* and rice (Takeda *et al*., 1987; Delrue *et al*., 1992). *In vitro* studies have shown that GBSSI elongates chains within amylopectin in isolated starch granules. Experiments in pea embryos, potato tubers and sweet potato roots have demonstrated the incorporation of [$^{14}$C]glucose from ADP[$^{14}$C]glucose mainly into long chains

of amylopectin (Baba *et al*., 1987; Denyer *et al*., 1996a). Thus, it is likely that GBSSI participates in the formation of amylopectin, but its precise role is still under study.

Many isoforms of GBSS exist, which vary in their location of expression. Expression of *GBSS1* is mostly confined to storage tissues. Alternatively, another isoform of GBSS, GBSSII is expressed mainly in non-storage tissues such as leaves, green stems etc. and assists in transient starch storage (Fujita and Taira, 1998; Nakamura *et al*., 1998; Vrinten and Nakamura, 2000). GBSSII in pea embryos is located both in the soluble phase and on granules (Denyer and Smith, 1992). GBSSII contains an extra serine rich domain in its N-terminus, and three consecutive proline residues near its C-terminus; which are absent in other GBSS isoforms. A second barley isoform, GBSSIb, has been identified that shares 96.5% identity with wheat GBSSII and 65.3% identity with barley GBSSI (Patron *et al*., 2002). As in wheat, this isoform is expressed in tissues such as the pericarp that accumulate starch transiently.

***Soluble starch synthases***

Plant organs vary greatly in the classes of starch synthases they possess and in the relative contribution of the classes to soluble starch synthase activity. For example, *in vitro* studies suggest that the primarily active enzyme in maize endosperm, pea embryos and potato tubers is SSI, SSII, and SSIII respectively, even though each species possesses genes for all three isoforms. In mature wheat endosperm, SSI, SSII and SSIII all are present in the soluble phase, while SSII partitions between granule-bound and soluble phases during grain development (Li *et al*., 1999). Different isoforms undertake specific functions for amylopectin synthesis. Mutational and antisense studies support this hypothesis, as suppression of a specific isoform results in structural alterations in amylopectin. This implies that SSs might not be substituting fully for the lost isoform.

The study of SS mutants in a number of systems has been helpful in the assignment of *in vivo* functions/ roles for the soluble starch synthase isoforms in starch synthesis. Maize SSI is responsible for initiating the synthesis of the shorter A and B1 chains of amylopectin, which is DP 6-15 (Guan and Keeling, 1998; Commuri and Keeling, 2001). It has been reported that SSI in rice is responsible for elongating short chains of DP 6-7 to DP 8-12 (Fujita *et al*., 2006).

SSII plays an essential role in the synthesis of intermediate-length branch-chains of amylopectin in cereal endosperm. Monocots possess two classes of SSII genes: SSIIa and SSIIb.

*In vitro* studies of the two SSII isoforms from maize reveal different substrate specificities and kinetic properties (Imparl-Radosevich *et al*., 2003). Additionally, tissue specificity also occurs in cereals. SSIIa is present in the endosperms while SSIIb is present in photosynthetic tissues. SSII isoforms are thought to have a similar function across species, as loss of SSIIa in monocots and SSII in dicots results in altered amylopectin chain length distribution, granule morphology and crystallinity. SSIIa plays a role in the synthesis of intermediate size glucan chains of DP 12-24 by elongating short chains of DP ≤ 10. Deficiency of starch granule protein-1 (SGP1) or SSIIa in wheat results in the depletion of chains of DP 11-25, while increase in chains of DP 6-10 (Yamamori *et al*., 2000). Japonica rice is deficient in SSIIa, which results in having more short chains (A and B1) and fewer long chains in its amylopectin than the Indica rice counterpart (Umemoto *et al*., 1999). Maize *Sugary2* starch, deficient in SSIIa, consists of fissured and lobed granules, having less crystalline structures (Perera *et al*., 2001). Barley *Starch excess-6* mutant, deficient in SSIIa, leads to novel starch phenotypes i.e. low amylopectin content with short branch chain length and relatively high amylose content (>70%) (Morell *et al*., 2003). The role of SSIIb is unknown as no mutants have been identified.

Impact of loss of SSIII appears to differ with the genetic background. SSIII mutation in maize is called *Dull1*. It results in amylopectin with enriched short branch-chains, and a substantial decrease in the number of long B-chains; the chain length of the long-branch chains however, increases (Wang *et al*., 1993). *Dull1* mutations lead to a subtle phenotype which is only conspicuous in *Waxy* backgrounds (Gao *et al*., 1998). The peak chain length of the long B-chains of *du waxy* amylopectin increases to DP 51 from DP 45 in the non-waxy maize starch (Jane *et al*., 1999). In potato, antisense suppression of *SS3* results in modified amylopectin chain length distribution and reduced starch synthesis (Edwards *et al*., 1999).

Sequences for SSIV appear in a wide range of higher plants in EST databases. To date, no mutants have been isolated with lesions in this gene but a possible role in granule initiation has been assigned for this class of SS in the process of starch biosynthesis (Tetlow *et al*., 2004a).

### 2.5.4 Branching of glucan chains by starch branching enzymes

Starch branching enzymes catalyze cleavage of α-1,4 linkages and transfer the free C-1 to react with the C-6 hydroxyl group of glucose-unit in another chain and form an α-1,6 branch

linkage. The branching enzymes, although mostly soluble, are also found among the minor proteins associated within the starch granule. It is reasonable to assume that these granule-bound branching enzymes are responsible for the low level of α-1,6 branches found in amylose (Ball *et al*., 1998). Soluble SBEs exist in multiple isoforms, which are tissue-specific and are expressed at different stages during grain development. Analysis of the primary amino acid sequences of higher plant SBEs reveals two major classes; SBEI in maize (also known as SBE B in pea) and SBEII in maize (also known as SBE A in pea). Both classes differ in protein structure, with SBEII containing an extra flexible N-terminal domain, which ends in two or three consecutive proline residues (Dry *et al*., 1992; Burton *et al*., 1995). Similar to starch synthases, this domain is thought to affect the relative partitioning between granule-bound and soluble phases.

In cereals, SBEII is further made up of two discrete gene products, *SBE2a* and *SBE2b* (Rahman *et al*., 2001). Different SBE isoforms exhibit different spatial and temporal expressions. *SBE1* and *SBE2a* are expressed in the endosperm, whereas *SBE2b* is expressed in endosperm as well as reproductive tissues (James *et al*., 2003). In addition to their difference in pattern of expression, SBE isoforms differ in their substrate specificities. SBEI uses amylose as substrate and transfers longer chains, while SBEII uses amylopectin as substrate and transfers shorter chains (Guan and Preiss, 1993; Takeda *et al*., 1993; Preiss and Sivak, 1998).

DNA hybridization and protein separation studies with chromosome engineered wheat showed that the genes encoding SBEI were located on the long arm of chromosome 7 (Morell *et al*., 1997; Rahman *et al*., 1997). Analysis of genes identified from *Triticum tauschii* demonstrated that there are multiple copies of the *BE1* gene present in the genome, only one of which corresponds to the endosperm expressed protein (Rahman *et al*., 1997; 1999). A cDNA for the endosperm-expressed *BE1* gene was also described (Repellin *et al*., 1997) and the expression of the *BE1* gene was characterized in further detail, demonstrating that the transcribed products of the BEI gene undergo alternate splicing (Båga *et al*., 1999). Suppression of SBEI activity in monocots and dicots leads to subtle differences in intermediate and long chain fraction of amylopectin irrespective of the reserve organ (Flipse *et al*., 1996; Blauth *et al*., 2002; Satoh *et al*., 2003). It thus indicates that SBEI plays a minor role in elongation of intermediate and long chain fraction of amylopectin, and that SBEIIa and/or SBEIIb do not compensate for the lost activity of SBEI.

SBEIIa mutant of maize was studied, and a clear phenotype in leaf starch and unaltered storage starch was found (Blauth *et al.*, 2001). Thus SBEIIa has been suggested to have a role in transient starch synthesis in leaf. Additionally, SBEIIa seems to have either a non-significant role in endosperm starch synthesis, or a minor role which is compensated by other SBEs.

The altered amylopectin structure in mutants that lack SBEIIb suggests a unique role for this isoform. SBEIIb mutants of maize and rice were designated '*amylose extender*' because they have an apparent increase in the relative proportion of amylose to amylopectin in the endosperm. However, it was later found out that rice *ae:wx* double mutants that lack amylose produce greatly elongated amylopectin chains that may previously have been mistaken for amylose (Nishi *et al*., 2001). Overall, a non-functional SBEIIb results in a higher content of long-chain fraction of amylopectin and >50% amylose. This confirms the indispensible role of SBEIIb in the formation of the amylopectin structure.

A model for sequential action of SBEs has been derived from *in vitro* analysis of heterologously expressed maize SBEs (Seo *et al*., 2002). Expression of three functional maize SBE genes in a yeast strain lacking the endogenous yeast glucan branching enzyme showed that SBEI was unable to act in the absence of SBEIIa or SBEIIb, and that SBEII may act prior to SBEI on precursor polymers.

### 2.5.5 Debranching of starch by debranching enzymes

Debranching enzymes have been postulated to play a role in amylopectin biosynthesis, by catalyzing the hydrolysis of α-1,6 linkages (Ball *et al*., 1996; Myers *et al*., 2000; Nakamura, 2002). Two groups of DBEs exist in plants; isoamylase-type and pullulanase-type (also known as limit dextrinase). Both the hydrolases are similar in amino acid sequence and predicted structure; they however differ in their substrate preference (Zeeman *et al*., 2004). Isoamylase-type DBEs act on denatured amylopectin, glycogen and amylolytic derivatives as substrates; while pullulanase-type DBEs prefer spread-out structures such as pullulan (a polymer of repeating α-1,6 linked maltotriosyl units), and limit dextrins. The two kinds of DBEs are located within the plastids, and are tissue-specific. Presence of isoamylase-type DBE has been demonstrated in maize endosperm (Yu *et al.*, 1998) and pea embryos (Zhu *et al.*, 1998). Active pullulanase-type DBEs have been detected in germinating seeds, leaves, and in developing endosperm of maize,

rice, barley and pea (Nakamura, 1996; Zhu *et al*., 1998; Beatty *et al*., 1999; Burton *et al*., 1999), indicating their role in amylopectin synthesis.

Multiple isoforms of isoamylases exist, which are involved in both, starch synthesis and degradation. In *Arabidopsis*, three genes encoding isoamylase-type proteins (ISA1, ISA2, and ISA3) and one gene encoding limit-dextrinase (LDA) are reported. However, ISA1 and ISA2 have been shown to be subunits of one hetero-multimeric ISA protein *in vivo* in Arabidopsis and potato (Hussain *et al*., 2003). Functionally, ISA1 and ISA2 are utilized during starch synthesis, whereas ISA3 and LDA function during starch (transient) degradation (Dinges *et al*., 2003; Zeeman *et al*., 2004).

Starch debranching enzyme deficient mutant in maize is known as *Sugary-1* (Pan and Nelson, 1984), as it accumulates a high content of highly branched water-soluble phytoglycogen (Morell *et al*., 1995). In addition to cereals, a similar mutation has been reported for algae (Moullie *et al*., 1996) and potato (Bustos *et al*., 2004). A very small amount of *Sugary1* activity is sufficient for the synthesis of near-normal quantities of amylopectin (Dinges *et al*., 2001). Barley *ISA1* mutants synthesize phytoglycogen and have profoundly defective granule structure and initiation. Mutant granules are small, irregular in shape, often compound, and are produced during only one wave of granule initiation rather than during two waves that normally occur. Wheat *ISA1* cDNA is expressed maximally in developing wheat grains and not at all in mature wheat grains (Genschel *et al*., 2002). Maize mutant *ZPU1* lacking pullunase-type DBE was studied, and a reduced rate of starch degradation in leaves was observed during the night (Dinges *et al*., 2003). Furthermore, this line displayed a reduced rate of starch mobilization in the endosperm of germinating grains and a reduced rate of seedling growth (Zeeman *et al*., 2004). Biochemical characterization of *ZPU1* indicates that it is subject to activation by changes in redox status and inhibition in the presence of high sugar concentrations (Wu *et al*., 2002).

Two different mechanisms for the mode of action of DBEs have been proposed. The ‘glucan-trimming model’ suggests that DBEs directly participate in amylopectin synthesis, selectively removing branches that are inappropriately positioned (Myers *et al*., 2000; Nakamura, 2002). Accordingly, DBE activity would be required for maintenance of the cluster structure of amylopectin, for the dense packing of linear chains, and for growing chains to crystallize onto granule structure. In addition, the glucan chains released by the DBE action on amylopectin can

be elongated by GBSSI to form the amylose fraction. Recent observations, which show that the surface of the immature granules contain numerous short chains, are consistent with this model, but do not provide direct evidence of DBE mode of action (Nielsen *et al*., 2002). The role of DBEs in the 'soluble glucans recycling model' is to participate in the degradation of short chain glucan molecules that may be produced by SS or SBE action, to prevent accumulation of highly branched soluble polymers at the expense of amylopectin formation (Zeeman *et al*., 1998; Smith, 2001). Once starch molecules are synthesized in the semi-crystalline granule structure, the molecules are no longer susceptible to DBE hydrolysis. Isoamylase-type DBE mutants have been observed to have a higher granule number, which could be due to the encapsulation of branched glucan chains, which could not be degraded due to the absence of DBE (Burton *et al*., 2002).

### 2.5.6 Starch degradation by accessory enzymes

Starch degradation is an important determinant of the net starch yield in plants. The process of starch degradation varies with the reserve organ, because mature endosperm is a non-living cell mass, while transient starch reserve organs like leaves are living tissues (Smith *et al*., 2005).

During the day, starch and sucrose are synthesized together as the products of photosynthetic carbon assimilation in leaves. While sucrose is transported to non-photosynthetic parts of the plant, starch is temporarily accumulated in the chloroplasts. During the subsequent night, starch in leaves is degraded to provide substrates for sucrose synthesis to allow continued export to non-photosynthetic parts for plant metabolism. The initiation of starch degradation takes place by breaking open the intact starch granule by hydrolytic enzymes, followed by starch debranching and subsequently hydrolysis of linear chains of starch by amylases. Debranching results in the cleavage of α-1,6 glucosidic bonds producing linear glucan chains. Then, α-1,4 glucosidic linkages are hydrolyzed by α-amylases producing mixture of linear and branched malto-oligosaccharides, and β-amylases removing maltose units, ultimately resulting in glucose, maltose, malto-triose and a range of branched limit-dextrins. Another mechanism for cleavage of α-1,4 glucosidic linkages at the non-reducing end is through the production of glucose-1-phosphate, the reaction mediated by starch phosphorylases (Tetlow *et al*., 2004b).

In cereal endosperm as well, the degradation of glucans released from starch granules proceeds via limit dextrinase, α- and β-amylase and α-glucosidase to maltose and glucose, which can enter the embryo. These enzymes are either synthesized within surrounding cell layers or mobilized within the endosperm as degradation proceeds (Smith *et al*., 2005). The α-amylase required for glucan degradation, is secreted by the living aleurone cells into the endosperm. They attack the pores on the surface of the granule forming pits. The glucans released from the ruptured granule, are broken down to simpler forms by other hydrolytic enzymes (Maeda *et al*., 1978; Fincher, 1989).

It has been recently reported that phosphorylation of starch is essential for its subsequent degradation. Within the starch granule, glucan chains of the amylopectin fraction form double helices, which pack themselves into crystalline lamellae. During starch degradation, the double helices at the granule surface become disrupted with the addition of phosphate groups at C-6 and C-3 positions, by the action of glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD), respectively (Fettke *et al*., 2009). The addition of phosphate unwinds the double helices, making them accessible to β- amylases and isoamylases for degradation (Kötting *et al*., 2010). A mutation in the Arabidopsis *GWD1* gene is known as the *Sex1* mutation. This mutant exhibits strongly reduced growth and accumulates nearly phosphate-free starch to very high levels (Yu *et al*., 2001).

Disproportionating enzyme (D-enzyme) is localized in chloroplasts (Okita *et al*., 1979; Lin *et al*., 1988) and acts during starch degradation. D- enzymes transfer a segment of one linear chain to another. An internal α-1,4 linkage is cleaved and the released reducing end is attached to a separate chain through a new α-1,4 linkage. In such a case, the transferred fragment could be a maltosyl residue or maltotriose. The acceptor molecule could be a glucose residue, malto-oligosaccharide or a polyglucan chain (Kakefuda and Duke, 1989; Takaha *et al*., 1993).

Starch phosphorylase has been long considered to play a role in starch degradation. Maize starch phosphorylase, Pho1, has been shown to possess a dominant phosphorolytic action over synthetic action, when incubated with malto-oligosaccharides (Mu *et al*., 2001).

R1 protein was discovered in potato, and has been postulated to play an important role in starch degradation. Antisense studies showed reduced phosphate content and reduced starch degradation in the absence of R1 (Lorberth *et al*., 1998).

### 2.5.7 Multi-enzyme complexes

Starch synthetic enzymes operate as multi-protein complexes. Placement of starch synthases, starch branching enzymes and debranching enzymes in a complex could be positively influencing substrate binding or enzymatic activity, which would subsequently affect amylopectin structure (Hannah and James, 2008). Additionally, individual components in a starch synthesizing enzymes-complex would experience conformational changes, leading to their altered kinetic properties. Previously, studies with isolated plastids have demonstrated pleiotropic effects of one starch synthesizing gene on another, suggesting the existence of multi-enzyme complexes, where mutation within a complex would alter the complex leading to a loss or reduction in its catalytic ability. Some examples are listed in Table 2.3.

It has been shown recently, that the formation of enzyme complexes is dependent upon their phosphorylation status. Assembly of starch-synthesizing protein complex occurs by phosphorylation, and disaggregation taking place by dephosphorylation (Tetlow *et al*., 2004b). For instance, SBEIIb and starch phosphorylase have been shown to co-immunoprecipitate with SBEI in a phosphorylation dependent manner. In addition, multi-subunit complexes possess at least one large protein which is essential for the function and assembly of the complex (Nakayama *et al*., 2002). Starch phosphorylase has been suggested to be that large component, which functions as a scaffold within the complex. Two multi-subunit complexes have been reported based on *in vivo* protein-protein interaction tests in yeast nuclei, immunoprecipitation and affinity purification using recombinant proteins as the solid phase ligand. These include complexes between SSIIa, SSIII, SBEIIa and SBEIIb; and SSIIa, SBEIIa and SBEIIb (Hennen-Bierwagen *et al*., 2008). SSIII contains a coiled-coil domain (Hennen-Bierwagen *et al*., 2009), which is known to be involved in protein-protein interactions. Complexes involving SSI, SSIIa and either SBEIIa or SBEIIb have also been reported. These complexes were observed at specific stages of development (Tetlow *et al*., 2008).

Catalytically active protein complexes involved in amylopectin biosynthesis indicate that the kinetic properties of branching enzymes are altered in higher ordered states, most likely dimers. The effect of loss of SBEIIb activity (*amylose extender* mutation) on interactions between starch biosynthetic enzymes in maize endosperm amyloplasts was studied. It was proposed that during amylopectin synthesis, SSI and SSIIa together form the core of a

Table 2.3 Pleiotropic effects of starch biosynthesizing genes

| Mutation | Plant System | Gene affected | Pleiotropic effects | Reference |
|---|---|---|---|---|
| *amylose extender (ae)* | Maize | SBEIIb | - Loss of SBEI activity<br>- Altered property of isoamylase-type DBE | Colleoni *et al*., 2003 |
| *zpu1-204* | Maize | Pullulanase-type DBE | - Loss of SBEIIa activity<br>- Reduction in β-amylase activity | Dinges *et al*., 2003 |
| *su1-st* | Maize | Isoamylase-type DBE | - Loss of SBEIIa activity | James *et al*., 1995;<br>Dinges *et al*., 2001 |
| *amylose extender (ae)* | Rice | SBEIIb | - Decrease in SSI activity | Nishi *et al*., 2001 |
| *sex 6* | Barley | SSIIa | - Abolished binding of SSI, SBEIIa, SBEIIb within the granule matrix | Morell *et al*., 2003 |
| *dull 1* | Maize | SSIII | - Increase in SSI activity<br>- Reduced SBEIIa activity | Boyer and Preiss, 1981; Singletary *et al*., 1997; Cao *et al*., 1999 |

phosphorylation-dependent protein complex, which recruits SBEIIb under normal conditions, and SBEI, SBEIIa and SP in the absence of SBEIIb (Liu *et al*., 2009).

Formation of such complexes would improve the efficiency of amylopectin synthesis on the whole, as the product of one reaction would serve as a substrate for the following reaction (substrate channeling), thus reducing the consumption of extra energy for transfer of glucan chains. Additionally, it could support the formation of a three-dimensional crystalline amylopectin structure, with the formation of clustered branch points, side chains of defined length and particular side-chain packing (Tetlow *et al*., 2004a).

## 2.6 Resistant starch

In humans, ingested starch and its derivatives are digested in several stages. As it enters the mouth, starch is broken down into shorter oligosaccharides by salivary α-amylase. In the gut, further hydrolysis of partially digested material is performed by pancreatic α-amylase. On the basis of degree of hydrolysis of food while passing through the gut, starch is classified into 'available' and 'non-available'. Available starch refers to that portion of starch which is broken down to give energy. Non-available starch is that portion of starch which does not get assimilated by the human digestive tract. The non-digestibility of starch could be attributed to indigestible cell walls, dense structure, presence of dietary fiber, phytic acid or food processing.

On the basis of extent of digestibility, starch is classified into 'digestible' and 'resistant'. Digestible starches are those which are hydrolyzed in the human gut, namely rapidly digestible starch (RDS) and slowly digestible starch (SDS). RDS is referred to that portion of starch which gets hydrolyzed into constituent glucose molecules within 20 minutes of incubation with hydrolytic enzymes. RDS mainly includes amorphous and dispersed starch and is found mainly in starchy foods cooked by moist heat, such as bread and potatoes. SDS is referred to that portion of starch which gets hydrolyzed within 120 minutes of incubation with hydrolytic enzymes. SDS consists of physically inaccessible amorphous starch. Resistant starch (RS), on the other hand, is referred to that portion of starch which is not hydrolyzed until 120 minutes of incubation with amylase and pullulanase. It is, however, fermented in the large intestine by gut micro-flora (Sajilata *et al*., 2006).

Resistant starch has several physiological benefits. Foods containing resistant starch metabolize 5-7 hours after consumption, compared with normally cooked starch which is digested immediately. This results in slower release of glucose into the blood stream, thus reducing the demand for insulin (Annison and Topping, 1994; Kendall *et al*., 2004). This is measured as Glycemic Index (GI), and lowering the GI would have a direct effect on reducing type II diabetes. Resistant starch unabsorbed in the small intestine passes on to the large intestine where it is fermented by the micro-flora. The fermentation reaction produces short chain fatty acids (SCFAs), butyrate being the most beneficial. In addition to its role in providing energy to the colon cells and nutrition to colonic epithelium (Mentschel and Claus, 2003), butyrate facilitates the reversal of neoplastic changes *in vitro* (Ferguson *et al*., 2000) and induces apoptosis of damaged cells (Mentschel and Claus, 2003).

Resistant Starch is a major component of dietary fiber (Buttriss and Stokes, 2008). It is naturally present in all starch-containing foods, but the quantity can vary depending on the amount and type of starch present, how the food was processed, how it was stored prior to consumption, and how it was ingested. Based on the mechanism that contributes to their indigestibility, resistant starch is divided into four subtypes.

RS1 is inaccessible to digestion because of the presence of cell walls or other indigestible components present in the food matrix. Examples include unprocessed grains, seeds, and legumes.

RS2 resists digestion due to its granular structure and molecular architecture. Starch is tightly packed in a radial pattern and shows B-type polymorphism. Examples for RS2 include green bananas, raw potatoes or high amylose corn. Although the digestible resistance of native starches can be altered by food processing, however, high-amylose maize starch retains its granular structure even under processing conditions, hence maintaining its resistance to digestion. It has been observed that as the amount of amylose in the starch granule increases, there is a corresponding elevation in the recorded dietary fiber and resistant starch content. RS2 starches can be a very useful means of incorporating a source of natural resistant granular starch and dietary fiber into a wide variety of food and pharmaceuticals (Brown, 2004).

RS3 is retrograded starch which is produced during food processing and is digestion resistant due to its crystalline structure. Its examples include processed foods such as baked

goods and cereals. During food processing, upon hydration, amylose leaches out of the starch granules and forms a random coil structure. Upon cooling then, the randomly coiled chains rearrange themselves as double helices, stabilized by hydrogen bonds. Upon further retrogradation, the double helices pack in a symmetrical hexagonal unit cell. Gelatinized starch has A-type crystallinity, which modifies into B-type crystallinity upon retrogradation, hence making the starch more resistant (Eerlingen *et al.*, 1993b). Water molecules (36 to 42 molecules per unit cell) in the B structure are located in fixed positions within a central channel formed by 6 double helices. The degree of polymerization (DP) of amylose also affects the yield of RS3; it rises with DP up to 100 and thereafter remains constant (Eerlingen *et al*., 1993a). A minimum DP of 10 and a maximum of 100 seem to be necessary to form the double helix (Gidley *et al*., 1995).

RS4 represents that group of resistant starches which have been chemically modified (cross-bonded with difunctional chemical reagents, ethers, esters etc.) in such a way as to interfere with the action of digestive enzymes and therefore decrease their digestibility. There are no natural food sources of RS4. Hence the concept of using modified starches for nutritional purposes as macronutrients in foods is novel and warrants careful consideration and assessment.

### 2.6.1 Factors affecting starch digestibility

Starch enzymatic hydrolysis and RS is influenced by several factors, both extrinsic and intrinsic properties of starch granules. Extrinsic factors comprise starch granule surface characteristics like porosity of granule and pit formation between the surface and centre of the granule (Fannon *et al*., 1992), or exo-corrosion (Gallant *et al*., 1997). Intrinsic properties of starch granules such as packing of amorphous and crystalline regions (Gallant *et al*., 1992; Zhang *et al*., 2006), interaction of amylose with other components such as lipids (Crowe *et al*., 2000), proteins (Escarpa *et al*., 1997) and/or enzyme inhibitors (Bjorck *et al*., 1987) also influence starch digestibility. Reduced digestibility of tuber starch granules has been attributed to their large and smooth surface along with their surface properties.

The amylose to amylopectin ratio is an important determinant of starch digestibility. Amylose has a linear chained structure, while amylopectin is highly branched with a higher number of free non-reducing ends than amylose. Thus, this difference in their structural

characteristics results in different digestibility patterns. The amylose to amylopectin ratio plays a major role in the formation of RS2 and RS3 (Sajilata *et al*., 2006). A positive correlation exists between amylose concentration and RS formation (Ito *et al*., 1999). The straight chains of amylose limit the access of small intestine α-amylases to the glucose units on amylose chain. Besides, terminal ends may not be accessible due to folding of a polymer. In contrast, the highly branched structure of amylopectin provides multiple glucose units that amylases can access readily. In high amylose starch, during cooking, starch granules are gelatinized and amylose leaches out forming random coils, which when cooled form double helices and hexagonal networks, which are resistant to digestion. On the other hand, in waxy starch aggregation of random coiled chains occurs, rather than the formation of crystalline networks, which makes them susceptible to digestion.

The intensity of starch digestion is also affected by degree of polymerization and/ or branching of glucan polymers, i.e. a reduction in the rate of hydrolysis with increased branching due to steric hinderance (Park and Rollings, 1994). Gamma irradiation generated rice mutants high in RS showed increased proportion of short chains with DP ≤ 12, decreased proportion of intermediate chains of 13 ≤ DP ≤ 36, and a decrease in long chains with DP ≥ 37 (Shu *et al*., 2007). Another report mentions that β-amylase and maltogenic α-amylase mediated partial reduction of outer branch chains of amylopectin reduces overall starch digestion rate, which is related to an increase in the amount of α-1,6 linkages and decreases in α-1,4 linkages (Ao *et al*., 2007). Changes in the amylopectin chain length distributions facilitate retrogradation to produce B- and V- type crystalline structures, leading to more resistant starch. It is generally believed that an increased proportion of longer chains makes the starch more resistant to digestion. A possible reason could be that longer chains form longer and more stable helices, which are further stabilized by hydrogen bonds, distributed over the entire crystalline region and cause decreased digestibility (Lehmann and Robin, 2007).

## 2.7 Starch biosynthesis in relation with resistant starch

### 2.7.1 ADP-glucose pyrophosphorylase

AGPase catalyzes the synthesis of ADP-glucose from ATP and glucose-1-phosphate. It is the first step in starch biosynthesis and AGPase is also a key regulatory enzyme in the starch

biosynthetic pathway. AGPase consists of two large and two small subunits, which affect allosteric and catalytic properties of the enzyme. Allosteric regulation of this enzyme plays a critical role in determining the amount of starch produced (Hannah and James, 2008). AGPase is allosterically activated by 3-phosphoglyceric acid (3-PGA) and inhibited by inorganic phosphate (Pi) in many plant tissues (Preiss and Sivak, 1996). It has been shown previously that manipulation of the sensitivity towards Pi leads to an increase in starch yield in various plant systems (Smidansky *et al*., 2002; Sakulsingharoj *et al.*, 2004; Wang *et al.*, 2007). In addition, AGPase activity is also regulated by the redox status (Hendriks *et al.*, 2003). In general, the active form of AGPase is present in the plastids of mature cereal tissues and sink tissues of non-cereal plants. Developing cereals however, differ with most of their AGPase activity localized mainly in the cytosol of endosperm cells. Specific transporters / ADP-glucose transporter channels are involved in the trafficking of the resultant ADP-glucose. In non-cereal plants, sucrose to starch pathway comprises plastid import of hexose phosphates which can be used in other biosynthetic processes in addition to starch synthesis. In cereals, on the other hand, plastid imported ADP-glucose or hexose phosphates are specifically utilized for starch synthesis (James *et al*., 2003).

AGPase being the key regulator of starch synthesis and ADP-glucose transporters being an important conductor of raw material for starch synthesis, mutations in both of these have been shown to dramatically affect the total starch content in maize, barley, pea and potato (Hylton and Smith, 1992; Shannon *et al*., 1998; Tjaden *et al*., 1998; Patron *et al*., 2004). The maize *Shrunken-2* and *Brittle-2* mutants have lesions in the large and small subunits of the cytosolic AGPase, respectively (Hannah and Nelson, 1976). *Shrunken-2* mutant kernels are deeply dented, with floury endosperm that has 25 % reduced starch but are sweet due to high sucrose concentrations (Hutchison, 1921). Similarly *Brittle-2* mutant kernels germinate poorly, are dark and shrunken and have 25-34% lower starch than non-mutant kernels (Preiss *et al.*, 1990). A barley mutant *Risø 16* is associated with a deletion in the small subunit of cytosolic AGPase resulting in reduced starch concentration and seed weight (Johnson *et al*., 2003). These changes in starch concentration have not been associated with RS.

### 2.7.2 Starch synthases

Starch synthases catalyze the transfer of the glucose unit from ADP-glucose to the non-reducing end of an already existing glucan chain by forming an α-1,4 glucosidic linkage. Cereal endosperms contain five classes of starch synthases, based on similarities in their conserved primary amino acid sequences. SSI and SSII are present mostly in the stroma (Fujita *et al*., 2006), whereas SSIII and SSIV are present both in the stroma and starch granule (Denyer *et al*., 1995; Dai, 2010) and are primarily involved in amylopectin synthesis. GBSSI is bound to starch granules and is required for amylose synthesis. Recently, GBSSI has also been shown to participate in the elongation of amylopectin chains, particularly for very-long branches (Yoo and Jane, 2002). The chain elongation pattern differs for each isoform and varies with plant species (Smith *et al*. 1997). In addition to their specific roles in amylopectin structure formation, some SS overlap in their functions (Roldán *et al*., 2007).

***Granule-Bound Starch Synthase I***

GBSSI (also known as waxy protein) present in the interior of the starch granule is essential for amylose synthesis. Plants lacking GBSSI enzymatic activity produce starch without amylose also called waxy starch. In wheat, GBSS has two isoforms GBSSI and GBSSII, the latter being present in the pericarp (Nakamura *et al*., 1998; Vrinten and Nakamura, 2000). In barley, another isoform called GBSSIb localized in the pericarp has been reported (James *et al*., 2003), which is important for enhancing the sink strength of the developing grain via transient starch accumulation (Patron *et al*., 2002). *In vitro* study using ADP[$^{14}$C]glucose as precursor of starch biosynthesis in isolated starch granules showed uptake of malto-oligosaccharides of DP 2-7 by GBSSI as primers for amylose synthesis (Denyer *et al*., 1996a). GBSSI is also reported to be involved in the elongation of long chains of amylopectin (Craig *et al*., 1998; Yoo and Jane, 2002). GBSSI elongation of amylose or long-branch-chains of amylopectin has been reasoned to be due to GBSSI's ability to elongate unbranched glucan chains within the semi-crystalline region, which ultimately remain super linear in the mature granule, and are called amylose or long-branch-chains of amylopectin.

*Amylose in relation to RS formation*: Amylose contributes to the formation of RS2 and RS3 in particular. Deficiency of GBSSI activity produces starch made of only amylopectin (waxy starch). Completely or partially waxy starches have been shown to possess higher

digestibility as compared to their non-waxy counterparts, in different plant systems (Rooney and Plugfelder, 1986; Bertoft *et al*., 2000; Li *et al*., 2004; Chung *et al*., 2006; Asare *et al*., 2011). In a recent study on starch structure and *in vitro* enzymatic hydrolysis using barley atypical amylose concentration starch, high poly-dispersity indices for non-waxy (1.4) and increased amylose starch genotype (1.25) compared to near (partially) waxy starch genotypes (0.33) was reported using atomic force microscopy. It was also concluded that energy requirement for gelatinization and hydrolysis of waxy starch is lower than non-waxy or high amylose starch (Asare *et al*., 2011). Waxy starches are more susceptible to hydrolytic enzymes compared to starch granules with significant amylose concentration. Three types of rice cultivars with varying amylose content for *in vitro* hydrolysis and glycemic index determination were investigated. Quicker, complete and significantly higher rate of starch hydrolysis for waxy and low amylose rice than for intermediate and high-amylose rice was reported (Hu *et al*., 2004). In a more practical approach for estimating RS contribution from amylose, high-amylose wheat flour was substituted for non-waxy wheat flour in bread-making and higher RS content in the substituted bread was observed (Hung *et al*., 2005). External pressures such as cooking can also increase the amylose content of starch, by extended cooling after cooking, ultimately leading to higher resistant starch (Blazek and Copeland, 2010).

***Starch synthase I***

In maize, SSI is responsible for extending shorter A and B1 chains up to a critical chain length making it unsuitable for its own catalysis (Commuri and Keeling, 2001). In rice, retrotransposon *Tos17* insertion mediated SSI-deficient mutant lines showed starch phenotype with decreased amylopectin chains of DP 8-12, but increased chains of DP 6-7 and 16-19. This suggests that SSI functions in generating DP 8-12 chains from shorter chains of DP 6-7 emerging from the branch point of A and B1 chains (Fujita *et al*., 2006). Amylose synthesis was not affected by this mutation and its effect on starch hydrolysis has not been reported.

***Starch synthase II***

In cereal endosperm, SSII synthesizes intermediate-length branch chains of amylopectin (see review by Jane *et al*., 2010). A triple null wheat line lacking starch granule protein-1 (SGP1) was produced and the protein was identified as SSIIa (Yamamori *et al*., 2000) which was observed to be homologous to maize SSIIa (Li *et al*., 1999). Lack of SGP1 showed amylopectin

with increased short chains of DP 6-10, and a decrease in intermediate chains of DP 11-25, and a concomitant increase in apparent amylose concentration (30.8 – 37.4%). In subsequent study (Yamamori *et al*., 2006) wheat lines lacking SGP1 showed an increase in resistant starch level (3.6%) compared to non-mutant wheat (0.02%). In a similar approach, wheat lines deficient in SSII A and B genome polypeptides, resulted in increased amylose (32%) starch as determined by HP-SEC analyses (Chibbar and Chakraborty, 2005). SSIIa deficient maize (*Sugary2* mutation due to insertion in SSIIa), genotypes showed an increase in abundance of short (DP 6-11), and medium (DP 13-25) chains. This mutation also resulted in an increase in apparent amylose concentration from 26% to 40% (Zhang *et al*., 2004). In rice, *japonica* type has a higher short to long chains ratio than *indica* type; *indica* rice has a higher amylose concentration than *japonica* rice (Umemoto *et al*., 1999; 2002).

In barley, a single base-pair transition mutation on chromosome 7H results in the inhibition of C-terminal translation of the active site of SSIIa. This mutation is known as *Starch-excess*, or *sex6* mutation (Morell *et al.*, 2003). The starch phenotype of this mutation is a dramatically increased amylose concentration (65-70%), which leads to higher RS content as well. Additionally, inactive SSIIa also changed the crystallinity from A-type to a mixture of B- and V-types. V-type crystallinity suggests the formation of amylose-lipid complexes, which are themselves resistant to digestion (Morell *et al.*, 2003). *In vivo* studies have also shown an increase in RS content and altered large-bowel SCFA in rats fed with SSIIa-deficient high-amylose barley cultivar, *Himalaya-292* (Bird *et al*., 2004).

Similar alterations in amylose content and amylopectin fine structure have been reported in SS2a-deficient starches from potato (Edwards *et al*., 1999) and pea (Craig *et al*., 1998). SSIIa mutation in pea, *rug5*, decreases intermediate length amylopectin chains (B2 and B3) and results in a higher (~35%) amylose concentration starch (Craig *et al*., 1998).

***Starch synthase III***

SSIII functions to synthesize long-B-chains of amylopectin. In maize, mutation in *SS3* causes a dull appearance of the starch; hence it has been named *Dull-1*. The other effects of the *dull-1* mutation include decreased content of long B-chains, enriched short branch chains and moderately increased amylose content (Wang *et al.*, 1993). The *SS3* mutation also has pleiotropic effects on *SS2* and *SBE2a*, and it is capable of altering endosperm starch structure (Gao *et al*.,

1998). The rice *SS3* mutation is known as *floury (flo)*, which gets its name from the floury-like appearance of the endosperm, bearing small and round starch granules (Ryoo *et al*., 2007). In rice *flo* mutant lines, amylopectin chains with DP ≥ 30 were reduced, suggesting that OsSSIIIa has a role in generation of relatively longer chains of amylopectin i.e. B2 and B3 to B4. In addition, a 2-4% increase in amylose content was also reported. *Arabidopsis* SSIII mutants AtSSIII1 and AtSSIII2 showed increased starch concentration compared to non-mutants suggesting a negative regulatory role of SSIII in biosynthesis of transient starch (Zhang *et al*., 2005). However, no report is available on the effect of SSIII mutation on starch digestibility.

***Starch synthase IV***

Although the specific function of SSIV is not clear, SSIV has been postulated to have a role in starch granule initiation, as *Arabidopsis* SSIV mutants showed a dramatic reduction in leaf starch content (Roldán *et al.*, 2007). Recently it has been shown in an *in vitro* assay that SSIV has high SS activity when malto-triose is used as primer (Szydlowski *et al.*, 2009). Two isoforms of SSIV, i.e. SSIVa and SSIVb expressed during grain filling in both pericarp and endosperm have been reported in rice (Hirose and Terao, 2004). To-date no cereal plants deficient in SSIV activity have been characterized.

### 2.7.3 Starch branching enzymes

Starch branching enzymes cleave α-1,4 linkages and transfer the free reducing C-1 to C-6 hydroxyl group of glucose-unit in another chain forming a new α-1,6 branch linkage. The available concentration of SBE is critical for the formation of amylopectin, as a lower concentration would result in lower branching. Based on primary amino acids sequence similarity and substrate specificity, two major types of SBE (SBEI and SBEII) have been identified in cereals. *In vitro* studies in maize suggest SBEI prefers amylose as substrate and transfers longer chains, whereas SBEII uses amylopectin as substrate and transfers shorter chains (Guan and Preiss, 1993). In wheat, SBEII is further divided into two ~85% similar isoforms SBEIIa and SBEIIb with apparently similar molecular weight (Rahman *et al*., 2001). Another isoform of SBE, SBEIc has been reported in wheat (Båga *et al*., 2000), which is associated with large A-type starch granules (Peng *et al*., 2000). In dicots like pea and potato, two isoforms of

SBE viz. SBEI and SBEII (or, SBE B and SBE A) have been reported (Poulsen and Kreiberg, 1993; Burton *et al*., 1995).

In maize, a mutation in SBEIIb resulting in high amylose starch is known as *Amylose-extender (ae)* (Stinard *et al*., 1993). It results in cereal starch with high amylose concentration (>50%), amylopectin with a higher number of long branch-chains and fewer short branch-chains (Jane *et al*., 1999). Similarly in rice, *ae* starch characteristics are enriched long chains of DP ≥ 38, reduced proportion of short chains of DP ≤ 17 and increased apparent amylose concentration (Nishi *et al.*, 2001). The very long chains of *ae* mutant amylopectin develop B-type crystallinity (Hizukuri *et al.*, 1983; Kasemsuwan *et al*., 1995), which favor slow enzymatic digestion. These results corroborated a similar study in maize (Li *et al*., 2008), where *ae* mutants showed a significant increase in chain lengths of amylopectin, and higher apparent and absolute concentration of amylose, which further resulted in higher RS content. Commercially, the *ae* mutation has been exploited to produce high-amylose (~80%) starch, which is called Hi-maize and it has been added to wheat products to increase RS content (Brown, 2004).

In a recent study in barley, RNAi mediated inhibition of SBEIIa and SBEIIb activity altered starch composition and structure (Regina *et al*., 2010). Down-regulation of individual SBEs resulted in subtle differences, however reduction in expression of SBEIIa+b resulted in a dramatic increase in amylose content, enriched amylopectin chains of DP < 9 and DP >15, and consequent reduction in the content of medium chains (DP 9-13). A similar trend has previously been reported in wheat where an increase in amylose content (< 70%) in SBEIIa mutants was observed by simultaneous inhibition of expression of both the SBE II isoforms (Regina *et al*., 2006). In addition, decrease in proportion of amylopectin chains of DP 4-12 and an increase in chains of DP >12, was also seen. *In vivo* feeding studies in rats using high-amylose wheat meal showed higher amount of RS and lower glycemic index in comparison to a non-waxy wheat diet (Regina *et al*., 2006). In other plant systems, for example in potato, inhibition of SBE A and SBE B has been shown to increase the amylose concentration and absence of high-molecular weight amylopectin, which would lead to lower digestibility of the starch (Schwall *et al*., 2000).

Corn starch with different doses of *ae* and *flo* alleles have been studied previously and highest amylose and RS contents were reported for *aeaeaeae*, as compared to all other combinations (Yao *et al*., 2009). The authors also observed a higher proportion of longer branch

chains with DP ≥ 25 in these mutants. Since the *amylose-extender* mutation reduces SBEIIb activity, it results in an increase in amylose to amylopectin ratio, which in turn increases RS content.

### 2.7.4 Starch debranching enzymes

Final packaging of starch granules requires trimming of extra branches. Debranching enzymes have been postulated to play this important role in amylopectin biosynthesis (Ball *et al*., 1996; Myers *et al*., 2000; Nakamura *et al.*, 2002). Absence of DBEs in a cereal endosperm results in the formation of soluble phytoglycogen instead of crystalline amylopectin (Zeeman *et al*., 1998). Two different mechanisms for DBE mode of action have been proposed. The 'preamylopectin-trimming model' suggests that the outer branches of preamylopectin molecules are trimmed by DBE to facilitate chain elongation by SS (Mouille *et al*., 1996; Myers *et al*., 2000). This will form amylopectin with an ordered branch structure and allow packaging of the molecule in starch granules. In addition, glucan chains released by DBE's action on amylopectin can be elongated by GBSSI to form the amylose fraction. According to the 'soluble glucan recycling model', DBE participates in degradation of short chain glucan molecules produced either by SS or SBE action to prevent accumulation of highly branched soluble polymers at the expense of amylopectin formation (Zeeman *et al*., 1998; Smith, 2001).

DBEs are divided into two major classes, based on the type of substrate they act on. First are isoamylases, which act on packed structures like amylopectin or glycogen. Second are pullulanses which attack more open structures like pullulan. Three isoforms of isoamylases have also been reported in cereal endosperm (Kubo *et al.*, 2005) and in potato (Hussain *et al*., 2003). Lack of isoamylase-1 in rice (*sugary-1,su-1),* and barley *(isa-1)* resulted in small but significant alteration in amylopectin chain length distribution (Kubo *et al*., 2005). Starch morphology is also altered, with starch granules being shrunken, irregular and compound (reviewed in James *et al*., 2003). Mutation in maize pullulanase is termed *ZPU1*, which is redox regulated and inhibited by high sugar (Dinges *et al.*, 2003). Similarly, redox regulation has also been reported for limit-dextrinase-type-DBE in wheat (Repellin *et al*., 2008). Since mutation in DBEs lead to the formation of phytoglycogen, it would automatically lower the digestibility of starch. Mutations in debranching enzymes, however, have not been reported to be associated with resistant starch.

## 2.8 Concluding remarks

Starch biosynthesis is a complex process in which starch biosynthetic enzymes act in a coordinated manner to produce amylopectin which is architecturally conserved in starches from different botanical sources. Specific function of each enzyme or its isoform has been deciphered by identifying lesion(s) in particular gene(s) encoding starch biosynthetic enzymes, and studying its effect on starch composition and structure. In addition, it has also been found that pleiotropic effects exist between starch biosynthetic genes. For example, mutation in the maize *SBE2b* gene reduces *SBE1* activity and affects properties of ISA as well (Colleoni *et al*., 2003). In contrast, genetic lesions in pullulanase or isoamylase reduce *SBE2a* activity, without altering the SBEIIa polypeptide (James *et al.*, 1995; Dinges *et al.*, 2003). Mutations in SSII reduce the binding of SSI, SBEIIa and SBEIIb within the granule matrix, without affecting their affinity towards amylopectin (Morell *et al*., 2003; Umemoto and Aoki, 2005). These instances suggest that starch biosynthetic enzymes exist/ operate as protein- complexes, which have been shown in wheat and maize (Tetlow *et al.*, 2004b; Hennen-Bierwagen *et al.*, 2008). In addition, phosphorylation of enzymes like GBSSI, SBEIIb and Pho1 is important for their incorporation into starch granules (Grimaud *et al*., 2008). The concept of starch biosynthetic enzymes acting in a complex and their formation dependent upon the phosphorylation status of constituent enzymes is an additional level of control in starch biosynthesis.

There is significant interest in increasing amylose concentration in cereal and tuber starches. Increased amylose concentrations have been attributed to both SBE and SS isoforms. In addition to natural mutants in maize (*ae*) and barley (*sex6*), amylose to amylopectin ratios in starch have been manipulated by altering GBSSI and SBEII (*waxy / amylsose extender*) activity in wheat (Regina *et al*., 2006; Lafiandra *et al*., 2010; Sestili *et al*., 2010), maize (Jiang *et al*., 2010) and rice (Wei *et al*., 2010a). In wheat and barley, very high amylose concentrations were obtained by RNAi mediated inhibition of *Sbe2a* and *Sbe2b* genes (Regina *et al*., 2006; 2010). Recent advances in understanding starch biosynthesis combined with innovations in genomics (Ganeshan *et al.*, 2010) can be used to develop cereal genotypes with increased amylose concentrations and alteration in amylopectin architecture which can be used to produce RS.

A summary of the relation of starch biosynthetic enzymes with relation to digestibility is shown in Table 2.4.

Table 2.4 List of known starch biosynthetic mutants with modified starch content and structure in relation to digestibility

| Enzyme | Genus | Mutant | Starch content and structure | Digestibility | Reference |
|---|---|---|---|---|---|
| **AGPase** | *Hordeum vulgare* | *lys5* | low starch content | not reported | Patron *et al*., 2004 |
| **AGPase** | *Pisum sativum* | *rb* | reduced starch content | not reported | Hylton and Smith, 1992 |
| **AGPase** | *Solanum tuberosum* | | ~10% decrease in amylose | not reported | Tjaden *et al*., 1998 |
| **GBSSI** | *Hordeum vulgare* | | ↑ amylose, ↑ DP 19-36 | ↓hydrolysis | Asare *et al*., 2011 |
| **GBSSI** | *Ipomoea batatas* | *wx* | amylose free; lesser short chains | ↑ hydrolysis | Noda *et al*., 2009 |
| **SSI** | *Oryza sativa* | | amylose unaffected; ↑DP 6-7,16-19; ↓DP 8-12 | not reported | Fujita *et al*., 2006 |
| **SSII** | *Hordeum vulgare* | *sex6* | amylose 65-70%; ↓short chains | ↓hydrolysis | Morell *et al*., 2003; Bird *et al*., 2004 |

| | | | | | |
|---|---|---|---|---|---|
| **SSII** | *Triticum aestivum* | | ↑ amylose 35%; ↓average CLD | resistant starch ↑100 fold | Yamamori *et al*., 2000 |
| **SSII** | *Pisum sativum* | *rug5* | ↓ B2,B3; ↑ very short and very long chains; ↑ amylose ~35% | not reported | Craig *et al*., 1998 |
| **SSIII** | *Zea mays* | *dull1* | ↑apparent amylose; ↑short chains; ↓long B chains | not reported | Gao *et al*., 1998 |
| **SBEIIa+b** | *Hordeum vulgare* | | amylose >65%; ↑DP <9,>15; ↓DP 9-13 | ↑ resistant starch | Regina *et al*., 2010 |
| **SBEIIb** | *Zea mays* | *ae* | ↑apparent and amylose content; ↑long chains | ↑resistant starch to ~40% | Li *et al*., 2008 |
| **SBEI, SBEIIa +b** | *Hordeum vulgare* | | amylose-only starch granules | ↑resistant starch to ~65% | Carciofi *et al*., 2012 |
| **DBE** | *Oryza sativa* | *su-1* | altered amylopectin CLD; granules shrunken, irregular and compound | not reported | Kubo *et al*., 2005 |

↑ - increase, ↓ - decrease

# 3. METHODS

## 3.1 Starch isolation

Starch was purified from mature wheat seeds using a previously described method (Peng *et al*., 1999) with slight modifications. In brief, coarsely ground 8-10 wheat seeds were steeped in 2 mL of water for 2 h, crushed, and filtered through a 100 μm pore-size nylon filter. The crude starch slurry was centrifuged at 4,000 g for 10 min. The pellet obtained was suspended in 200 μL of water, layered over 1 mL 80% (w/v) cesium chloride and starch was isolated using density gradient centrifugation at 13,000 g for 30 min. The crude starch pellet was washed once with water, followed by one wash with 500 μL wash buffer {55 mM Tris-HCl pH 6.8, 2.3% (w/v) sodium-dodecyl sulphate, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol}, subsequently two water washes, each at 13,000 g for 10 min. Purified starch was finally treated with acetone and air-dried overnight. The procedure resulted in ~70% starch recovery efficiency.

## 3.2 Total starch concentration

Total starch concentration was determined using the AACC approved method (AACC method 76-13.01). In brief, ~100 mg (duplicates) ground meal samples, suspended in 0.2 mL 80% (v/v) ethanol, were incubated with 3 mL α-amylase (240 U/mL, Megazyme International Ltd., Wicklow, Ireland) in 100 mM sodium acetate buffer pH 5.0 at 100°C for 6 min with intermittent mixing every 2 min to hydrolyze starch to maltodextrins. Subsequently, the samples were incubated with 0.1 mL 330 U amyloglucosidase (3300 U/mL, Megazyme International Ltd., Wicklow, Ireland) in 200 mM sodium acetate buffer pH 4.5 at 50°C for 30 min, to hydrolyze maltodextrins to glucose. After the reaction was complete, sample volume was made up to 10 mL with water and three aliquots (10 μL each) were transferred into different tubes with 3 mL of glucose determination reagent, GOPOD. Samples and glucose standard were incubated at 50°C for 20 min. Total starch concentration was determined as free glucose by measuring the absorbance at 510 nm (Hucl and Chibbar, 1996) using a spectrophotometer (DU 800, Beckman Coulter Inc., Mississauga ON, Canada). Starch concentration was calculated on a percentage dry weight basis (McCleary *et al*., 1997).

Starch (%) = ΔA x F x FV/0.01 x 1/1000 x 100/W x 162/180

where,

ΔA = Absorbance (reaction) read against the reagent blank

F = $\frac{\text{100 (µg of D-glucose)}}{\text{Absorbance for 100 µg of glucose}}$ (conversion from absorbance to µg)

FV = Final volume i.e. 10 ml

0.01 = volume of sample analyzed

1/1000 = Conversion from µg to mg

100/W = Factor to express starch as a percentage of dry flour weight

W = weight (in mg) of flour analyzed

162/180 = Adjustment from free D-glucose to anhydro D-glucose (as occurs in starch)

## 3.3 Protein concentration determination

Protein concentration was determined with FP-528 Protein/Nitrogen Analyzer (LECO Corporation, St. Joseph MI, USA), where nitrogen generated by pyrolysis is combusted at high temperature and further quantified by thermal conductivity detection. Meal samples (0.25 g ± 0.01 g) in duplicates were combusted for protein analysis. Percentage protein concentration of samples was obtained using the formula: P (%) = N (%) x C, where C is 5.7 for wheat (AACC method 46-30.01).

## 3.4 Crude lipid concentration determination

Crude lipid concentration was determined by ANKOM$^{XT15}$ extractor (ANKOM Technology, Macedon NY, USA) using petroleum ether as the extraction solvent (AACC method 30-25.01). Briefly, 1 g wheat meal was weighed in filter (Whatman #2) paper bag, oven-dried at 130°C for 3 h, cooled in desiccant, weighed again and placed in the carousel of the lipid extractor. The extraction was carried out using hexane at 105°C for 120 min. After extraction, samples were kept at 100°C for 15-30 min, cooled in desiccant and re-weighed. Percent lipid was expressed as weight of lipid per gram dry weight of initial material used.

$$\text{Lipid (\%)} = 100 \times \frac{\text{(Wt. after drying – Wt. after extraction)}}{\text{Wt. sample}}$$

## 3.5 Beta-glucan concentration determination

Beta-glucan concentration was determined using enzymatic method (AACC method 32-23.01). Wheat meal (~100 mg) was suspended in 1 mL 50% (v/v) ethanol, mixed with 4 mL 20 mM sodium phosphate buffer pH 6.5 and boiled for 5 min with intermittent mixing. After cooling, samples were digested with 200 U lichenase (1000 U/mL, Megazyme International Ltd., Wicklow, Ireland) for 1 h at 50°C. Further, 5 mL 200 mM sodium acetate buffer pH 4.0 was added, vigorously mixed and centrifuged at 1,000 g for 10 min. An aliquot (0.1 mL; triplicates) of the supernatant was treated with 4 U of β-glucosidase (40 U/mL, Megazyme International Ltd., Wicklow, Ireland) in 50 mM sodium acetate buffer pH 4.0 and incubated at 50°C for 10 min. 3 mL of glucose determination reagent (GOPOD) was added and tubes incubated at 50°C for 20 min. Mixed linkage β-glucan was calculated as free glucose (McCleary and Codd, 1991) by measuring the absorbance at 510 nm using a spectrophotometer (DU800, Beckman Coulter Inc., Mississauga ON, Canada).

$$\beta\text{-glucan (\%)} = \Delta A \times F \times 94 \times 1/1000 \times 100/W \times 162/180$$

where,

$\Delta A$ = Absorbance after β-glucosidase treatment (reaction) minus reaction blank absorbance

$F = \frac{100\ (\mu g \text{ of D-glucose})}{\text{Absorbance for } 100\ \mu g \text{ of glucose}}$ (conversion from absorbance to μg)

94 = Volume correction factor (0.1 ml out of 9.4 ml was analyzed)

1/1000 = Conversion from μg to mg

100/W = Factor to express β-glucan as a percentage of dry flour weight

W = Dry weight (in mg) of flour analyzed

162/180 = Adjustment from free D-glucose to anhydro D-glucose (as occurs in β-glucan)

## 3.6 Amylose concentration determination

High-performance size exclusion chromatography (HP-SEC) was used to determine the amylose concentration, as described previously (Demeke *et al*., 1999). Starch suspension was prepared by adding 5 mL dd$H_2O$ to 5 mg starch and heating it at 130 °C for 30 min, with intermittent mixing. Further, 1 mg/mL of starch suspension was incubated with 55 µL 1 M sodium acetate buffer pH 4.0 and 4 U isoamylase (200 U/mL, Megazyme International Ltd., Wicklow, Ireland) to debranch starch. After debranching for 4 h at 40°C, the reaction was terminated by boiling samples for 20 min, the samples thereafter freeze-dried. Freeze-dried sample was suspended and dissolved in 200 µL 99% (v/v) dimethyl sulfoxide (DMSO) and centrifuged at 13,000 g for 10 min. A 40 µL aliquot of the supernatant was injected into a PL gel 5 µM MiniMix- C guard column attached to a PL gel MiniMix 250 x 4.6mm ID column (Polymer Laboratories Inc., Amherst MA, USA) to separate amylose and amylopectin using a high performance liquid chromatography (HPLC) system comprising a Waters 600 controller, Waters 610 fluid unit, Waters 717 plus autosampler and Waters 410 differential refractive index detector (Waters Corporation, Milford MA, USA). Starch samples, column and detector were maintained at 40, 100 and 40°C respectively. The eluent used was 99% (v/v) DMSO with 4.4% (w/v) lithium bromide at a flow rate of 0.35 mL/min, with 20 min injection and 30 min delay time. The data was collected and analyzed using Empower 1154 chromatography software (Waters Corporation, Milford MA, USA). Amylose concentration was calculated by integration of the peak area corresponding to amylose with respect to the peak area corresponding to amylopectin.

## 3.7 Starch granule size distribution analysis

Granule size distribution (by volume) of starch suspension was determined using a laser diffraction particle size analyser (Hydro 2000S, Mastersizer 2000, Malvern Instruments, Malvern WR, UK) (Asare *et al*., 2011). Starch solution (20 mg/mL) was used for size analysis at the laser obscuration of 11-12% and pump speed of 1,700 rpm. Volume-percentage particle size distribution values were obtained using Malvern Mastersizer 2000 software (Malvern Instruments, Malvern WR, UK).

## 3.8 Amylopectin chain length distribution analysis

Chain length distribution of amylopectin molecules was determined by fluorophore-assisted capillary electrophoresis (FACE) (O'Shea *et al*., 1998) using the Proteome Lab PA800 (Beckman Coulter, Fullerton CA, USA) equipped with a 488 nm laser module. A modified debranching protocol (Asare *et al*., 2011) was used to obtain unit amylopectin chains. Purified, defatted starch sample (20 mg) was suspended in 750 µL water, followed by addition of 50 µL 2 M NaOH. The suspension was mixed vigorously and boiled for 5 min, then cooled at room temperature and neutralized with 32 µL glacial acetic acid. Further, 100 µL 1 M sodium acetate buffer pH 4.5, 1 mL water and 10 U isoamylase (1000 U/mL, Megazyme International Ltd., Wicklow, Ireland) were added and mixed vigorously. The solution was then incubated at 37°C for 2 h. The reaction was terminated by boiling the reaction mixture for 10 min. Debranched starch was deionised by filtering through resin (20-50 mesh size) in a microfuge tube. After deionisation, 50 µL aliquot was dried for 2 h under vacuum (SPD SpeedVac, Thermo Electron Corporation, Milford MA, USA). Thereafter 5 µL maltose (internal standard) was added to the dried sample and dried again under vacuum. Released starch chains were fluorescent labelled overnight with 2 µL 8-aminopyrene 1,2,6-trisulfonate (APTS) which labels one molecule of oligosaccharide per molecule of APTS, in the presence of 2 µL of 1 M sodium cyanoborohydride / tetrahydrofuran. Labelling reaction was terminated by adding 46 µL deionised water, mixed, centrifuged at 13,000 g for 10 min and 40 µL supernatant was pipetted out, out of which 5 µL plus 195 µL deionised water was used for analysis. Debranched samples were separated using N-CHO (PVA) capillary with pre-burned window (50 µm ID and 50.2 cm total length). Samples (stored at 11°C) were injected at 0.5 psi for 3 sec and separated at constant voltage of 30 kV for 30 min. Data was recorded and analysed using 32 Karat software (Beckman Coulter, Fullerton CA, USA). The degree of polymerization was assigned to peaks based on relative migration time of maltose used as an internal standard. On the basis of changing slopes of the curve, amylopectin unit chains were divided into seven regions: R-I (DP 6-8), R-II (DP 9-11), R-III (DP 12-14), R-IV (DP 15-18), R-V (DP 19-22), R-VI (DP 23-36), and R-VII (DP 37-45).

## 3.9 Starch components morphology

Starch solutions were dissolved in water to a concentration of 1 mg/mL by heating the mixture to 95 °C for 5 min. Thereafter, it was diluted to 30 µg/mL concentration and kept at 90

°C. Approximately 10 µL of dissolved starch was deposited onto freshly cleaved mica as an aerosol spray by nitrogen gas. AFM images were taken using a PicoSPM instrument (Molecular Imaging, Tempe AZ, USA) which operates in intermittent contact mode. The force constant on the silicon cantilever (Nanoscience Instrument, Phoenix AZ, USA), the resonant frequency and the curvature radius for AFM imaging were 48 $Nm^{-1}$, 190 kHz and < 10 nm respectively. The ratio of set-point oscillation amplitude to free air oscillation amplitude was 0.75:0.85 while resonance amplitudes ranged from 0.4 to 1.0 V (Maley *et al*., 2010). The instrument was under ambient conditions and mounted in a vibration isolation system with a scan rate 1-1.5 Hz (512 pixels per line). Analysis of images and measurements were done using SPIP V5.0.5 software (Image Metrology, Lyngby, Denmark).

### 3.10 Starch granule morphology

Prior to imaging under scanning electron microscope, isolated starch granules were gold coated to make them electrically conductive. Starch granules were spread/ attached to a metal stub with double-sided carbon tape and gold coated using Gold Sputter Coater (S150B, Edwards, Wilmington MA, USA). Granule morphology was observed using scanning electron microscope (SU6600, Hitachi High-Technologies Europe GmbH, Krefeld, Germany). For taking energy dispersive spectroscopy (EDS) scans, the working distance was 10 mm at 5 kV. Aztec software was used as the post-processing software for gathering line and map scans.

### 3.11 Starch granule protein analysis

Proteins were extracted from the starch granules by suspending 10 mg starch in 300 µL treatment buffer {0.5 M Tris-HCl pH 6.8, 10% (w/v) sodium-dodecyl-sulfate, 10% (v/v) glycerol, β-mercaptoethanol and bromophenol blue}. Starch suspension was boiled for 5 min, cooled for 5 min and centrifuged at 12,500 g for 12 min. Starch granule-bound proteins were thus obtained in the supernatant. GBSSI was separated on one-dimensional denaturing polyacrylamide gel electrophoresis, as described (Demeke *et al*., 1997). The 10% resolving gel contained 30:0.1% acrylamide/ bis-acrylamide, 1.5 M Tris-HCl pH 8.8, 50% (v/v) glycerol, 10% (w/v) sodium-dodecyl-sulfate, 10% ammonium persulfate, and 0.05% N,N,N',N'-tetramethylethylenediamine (TEMED). The 5% stacking gel contained 30:0.1% acrylamide/ bisacrylamide, 0.25 M Tris-HCl pH 6.8, 10% (w/v) sodium-dodecyl-sulfate, 10% ammonium

persulfate and 0.05% TEMED. Running buffer contained 24.22 g Tris-base, 115.36 g glycine and 8 g sodium-dodecyl-sulphate. 60 µL of the extracted protein was loaded and the gel was run for ~15 hrs at 10 mA. Separated polypeptides were visualized by silver staining (Gromova *et al*., 2006). Stacking gel was removed and the resolving gel was soaked in fixative (50% ethanol, 12% glacial acetic acid and 0.05% formalin) for ~2 hrs. The gel was then washed with sensitizing solution (0.02% sodium thiosulfate) for 2 min followed by a quick wash with deionised water. It was then washed with cold silver staining solution (0.2% silver nitrate and 0.076% formalin) for 20 min for effective binding of silver ions to proteins. The gel was briefly washed with deionised water. Protein bands were developed by agitating the gel in 200 mL developing solution (6% sodium carbonate, 0.0004% sodium thiosulfate and 0.05% formalin) for 2-5 min. The reaction was terminated using 12% acetic acid.

## 3.12 Immuno-detection of polypeptides on gels

After equilibrating the gel in transfer buffer for 30 min, starch granule-bound proteins separated on denaturing polyacrylamide gel electrophoresis, were transferred onto nitrocellulose transfer membrane (Protan $^{R}$, Whatman) at 10 V for 4 hrs (Gao and Chibbar, 2000). Proteins on the membrane were blocked with 200 mL blocking buffer for 4 x 30 min. After blocking, the membrane was incubated with primary antibody against the desired starch bound protein for 2.5 h with slow agitation. Antibodies against different starch biosynthetic enzymes were diluted to different concentrations, viz., GBSSI 1:2000, SSI 1:5000, SSII 1:5000, SBEI 1:5000, SBEII 1:5000. The membrane was then washed with 200 mL blocking buffer for 4 x 15 min, followed by incubation with secondary antibody (goat anti-rabbit alkaline phosphatise-conjugated antibody) diluted to 1:5000 for 1.5 h. This was again followed by 4 x 15 min washings with blocking buffer to remove excess secondary antibody. Following these washings, the membrane was washed with 200 mL New Buffer for 3 x 15 min. The antigen-antibody complex was detected using BCIP/NBT immunoscreening color development kit (Biorad, Hercules CA, USA). 30 mg Nitro blue tetrazolium chloride (NBT) was dissolved in 1 mL 70% dimethylformamide solution, and 15 mg 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (BCIP) was dissolved in 1 mL 100% dimethylformamide; both solutions thereafter dissolved in 100 mL colour development solution. Incubating the membrane with this solution under dark for

5-10 min confirmed the presence of proteins of interest. The marker lane was stained/ destained using amido black dye.

The composition of the buffers used is as follows:

Transfer buffer, pH 7.4 = 40 mM Tris base, 20 mM NaAc x $3H_2O$, 2 mM EDTA, 20% (v/v) Methanol, 0.05% (w/v) Sodium-doceyl-sulphate

10 X Phosphate buffered saline (PBS), pH 7.4 = 1.37 M NaCl, 26.8 mM KCl, 100 mM $Na_2HPO_4$, 17.6 mM $KH_2PO_4$

Blocking buffer = 5% (w/v) low-fat Carnation milk in 1 x PBS, 0.1% Tween 20

New buffer = 50 mM Tris-HCl pH 7.5, 150 mM NaCl

Color development solution = 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$

Amido black stain = 0.1% Amido black, 25% (v/v) Isopropnanol, 10% (v/v) Acetic acid

Amido black destain = 25% (v/v) Isopropanol, 10% (v/v) Acetic Acid

## 3.13 *In vitro* kinetics of enzymatic starch hydrolysis

Wheat meal and pure starch samples were enzymatically hydrolyzed *in vitro* for kinetic analysis, using a modified AACC approved method (AACC method 32-40.01). White bread, wheat meal and pure starch samples (100 mg, in triplicates) were incubated with constant agitation in a 4 mL solution of pancreatic α-amylase (10 mg/mL) and 3 U amyloglucosidase (3000 U/mL, Megazyme International Ltd., Wicklow, Ireland) in sodium maleate buffer (100 mM pH 6.0). For kinetic analysis of starch hydrolysis, reaction mixtures were incubated for 0, 30, 60, 90, 120 and 240 min at 37 °C, during which non-resistant starch was solubilized and hydrolyzed to D-glucose. A 1 mL aliquot was taken from the tube after each incubation and reaction terminated with an equal volume of 99% (v/v) ethanol. After amylolysis termination, the reaction mixture was centrifuged (3,000 g, 10 min) and the residue (isolated RS) was washed twice with 750 μL 50% (v/v) ethanol. Supernatants were decanted in separate tubes. The final residue after the centrifugations was dissolved in 250 μL 2 M potassium hydroxide by vigorously stirring on an ice-water bath over a magnetic stirrer for 20 min. This solution was neutralized with 800 μL 1.2 M sodium acetate buffer pH 3.8 and quantitatively hydrolyzed to glucose with 33 U amyloglucosidase (3300 U/mL, Megazyme International Ltd., Wicklow, Ireland) by

incubating at 50 °C for 30 min. Reaction volume was made up to 10 mL with water and centrifuged at 3,000 g for 10 min. 3 mL of glucose determination reagent (GOPOD) was added to 100 µL of the supernatant and incubated at 50°C for 20 min. Resistant starch concentration was determined as free glucose by measuring its absorbance at 510 nm using a spectrophotometer (DU800, Beckman Coulter Inc., Mississauga ON, Canada). Non-resistant starch was determined by pooling the earlier supernatants from each interval and making up the volume to 12.5 mL with 100 mM sodium acetate buffer pH 4.5. A 0.1 mL aliquot of this solution was incubated with 10 µL of 300 U amyloglucosidase (3300 U/mL, Megazyme International Ltd., Wicklow, Ireland) in 100 mM sodium maleate buffer pH 6.0 at 50 °C for 20 min. 3 ml of glucose determination reagent (GOPOD) was added to 100 µL of the reaction mixture and incubated at 50°C for 20 min. Soluble starch concentration was determined as free glucose by measuring its absorbance at 510 nm using a spectrophotometer (DU800, Beckman Coulter Inc., Mississauga ON, Canada). Rate of starch digested (hydrolyzed) was expressed as percent of total starch at the end of each interval. RDS, SDS, and RS were measured as the amount of starch hydrolyzed within 30 min, 120 min, and more than 120 min during the assay, respectively.

Hydrolysis index (HI) of grain meal and extracted starch was calculated using a non-linear model (Goñi *et al*., 1997; Frei *et al*., 2003). The first order equation used is as follows: $C = C\infty (1 - e^{-kt})$ where, *C* corresponds to the percent starch hydrolyzed at time *t*, *C*∞ is the equilibrium concentration of starch hydrolyzed after 240 min, *k* is the kinetic constant and *t* is the selected time (min). The parameters *C*∞ and *k* were estimated for both meal and pure starch of all the lines, based on the data obtained from the *in vitro* hydrolysis procedure (Goñi *et al*., 1997). The area under the hydrolysis curve (AUC) was calculated using the equation: $AUC = C\infty (t_f - t_o) - (C\infty/k) [1 - \exp \{-k (t_f - t_o)\}]$ where, *C*∞ corresponds to the equilibrium concentration of starch hydrolyzed after 240 min, $t_f$ is the final time (240 min), $t_0$ is the initial time (0 min) and k is the kinetic constant. Hydrolysis Index was calculated by dividing the area under the hydrolysis curve of each sample by the corresponding area of the reference sample (fresh white bread) (Frei *et al*., 2003).

## 3.14 Statistical analyses

All determinations were done in triplicate. The analysis of variance (ANOVA) of the means and Pearson's correlation were performed with SPSS V. 19.0 software (SPSS Inc.,

Chicago IL, USA). Multiple means comparisons were determined with Tukey's multiple range test at $p < 0.05$ confidence level. Dendrograms were prepared using Minitab V. 16.0 software (Minitab Inc., State College PA, USA).

# 4. DIFFERENCES IN STARCH GRANULE COMPOSITION AND STRUCTURE INFLUENCE *IN VITRO* ENZYMATIC HYDROLYSIS OF GRAIN MEAL AND EXTRACTED STARCH IN TWO CLASSES OF CANADIAN WHEAT (*Triticum aestivum* L.)

**Study 1**[1]

This study was conducted first in the series of the three studies included in this thesis. The data obtained from this study gave an idea for the range of values to expect in the subsequent studies. Specifically, this study analyzed various wheat grain and starch components which affect overall *in vitro* starch digestibility in six established Canadian wheat cultivars. It was found that small starch granules, amylose concentration and long chains of amylopectin were the major determinants of the rate of *in vitro* starch enzymatic hydrolysis. Broadly, these results might be helpful in altering the end-uses of wheat grains of these cultivars.

[1] **Ahuja, G.**, Jaiswal, S., Hucl, P., & Chibbar, R.N. (2013). Differences in Starch Granule Composition and Structure influence *In Vitro* Enzymatic Hydrolysis of Grain Meal and Extracted Starch in Two Classes of Canadian Wheat (*Triticum aestivum*). *Cereal Chemistry* (Revision under process).

## 4.1 Abstract

Starch granule composition and amylopectin structure affect starch digestibility, an important factor influencing wheat grain utilization for human food consumption. Six bread wheat cultivars with four belonging to the Canada Western Red Spring (CWRS) and two Canada Prairie Spring Red (CPSR) market classes respectively were analyzed for relationship between their grain constituents and *in vitro* enzymatic hydrolysis of starch. CPSR cultivars had higher grain weight, starch and amylose concentration compared to CWRS cultivars, which had higher protein concentration. Starch granule volume percent did not differ among the genotypes, except AC Foremost, which had a significantly ($p < 0.05$) higher volume percent of B-type starch granules (~15%) and lower volume percent of A-type starch granules (~9%) compared to other cultivars. Fluorophore assisted capillary electrophoresis (FACE) revealed lower content of R-IV (DP 15-18, ~6%) and higher content of R-VII (DP 37-45, ~7%) chains in the CPSR cultivars compared to the CWRS cultivars. *In vitro* enzymatic starch hydrolysis showed that compared to CWRS cultivars, the two CPSR cultivars had reduced amounts of readily digestible starch (RDS), and higher amounts of slowly digestible starch (SDS) and resistant starch (RS). Consequently, the two CPSR cultivars also showed lower hydrolysis index (HI) in grain meal as well as extracted starch. CPSR cultivars, with higher starch and amylose concentration, higher content of long chains of amylopectin showed reduced *in vitro* starch enzymatic hydrolysis rate.

## 4.2 Introduction

Wheat (*Triticum aestivum* L.) is a major cereal grain which is consumed in diverse and ethnically determined forms around the world. In the human diet, cereal grains including wheat contribute to almost half of the caloric intake. Excessive calorie intake (11.3 – 12.5 MJ/day) combined with a less active lifestyle causes weight gain and obesity (Swinburn *et al*., 2004). In Canada, the obesity rates increased from 16.0% to 19.8% in men and from 14.5% to 16.8% in women between 2003 and 2011 (http://www.statcan.gc.ca/daily-quotidien/120619/dq120619b-eng.htm). Obese persons are more prone to diabetes, high blood pressure and cardiovascular diseases. Therefore, changes in diet and / or alteration in wheat grain constituents that can reduce the caloric intake can help to alleviate diet associated ailments.

A wheat grain consists of three parts. The bran, which is the outermost layer of the kernel, is rich in vitamins and minerals. Bran also contains considerable amounts of phenolic compounds with anti-oxidant activity (Verma *et al*., 2008). About 80% of the kernel weight is made up by the endosperm that primarily consists of starch along with traces of lipids (0.1 – 1.0%) and proteins (0.05 – 0.5%). The germ or embryo lies at one end of the grain and represents only about 2% of the kernel. Germ is a rich source of vitamin B and E, oils and natural plant fat (Bechtel *et al*., 2009). In a wheat grain, starch is the major storage constituent and contributor to calories in the human diet. Starch is stored as water insoluble granules made up of one-quarter amylose and three-quarters amylopectin, along with trace amounts of proteins, lipids and ash. Amylose is a predominantly linear glucan polymer composed of α-1,4 linked glucose residues, with few branches (one branch per ~ 1000 residues). Amylopectin, on the other hand, is a highly branched glucan polymer with 4-5% of α-1,6 glucosidic linkages, with 20-25 glucose units between branch points (Hizukuri, 1985). Starch biosynthesis is a complex process and requires the participation of several starch biosynthetic enzymes and their isoforms, and their genes are spatially and temporally expressed (Zeeman *et al*., 2010). Amylose is primarily synthesized by granule-bound starch synthase I (GBSSI) (Shure *et al*., 1983) and it has also been associated with the extension of amylopectin chains (Ral *et al*., 2006). Wheat being an allohexaploid (A, B and D genomes) has three GBSSI isoforms, which differentially influence amylose synthesis (B > D > A), with the GBSSI-B suggested to be the most effective in amylose synthesis (Miura and Sugawara, 1996). In Australian wheat cultivars, inactive GBSSI-B has been associated with increased flour swelling volume and slightly reduced amylose concentration (Zhao *et al*., 1998).

Physiochemical properties and commercial end-use of starch is influenced by amylose to amylopectin ratio and amylopectin structure (Jobling, 2004). With the recent emphasis on dietary caloric intake, alteration of starch composition and structure that can cause a reduction in starch hydrolysis, is gaining importance. Higher amylose concentration and/or alterations in the amylopectin chain length distribution have been shown to reduce the rate of starch hydrolysis and hence reduction in caloric intake (Lehmann and Robin, 2007).

The Canadian Grain Commission classifies western Canadian wheats into eight market classes based on end-use (https://www.grainscanada.gc.ca/wheat-ble/classes/classes-eng.htm#b). CWRS is the predominant market class accounting for 88% of spring common wheat production in 2012 (http://www.agr.gc.ca/pol/mad-dam/index_e.php?s1=pubs&s2=go-

co&s3=php&page=go-co_2012-09-18), followed by CPSR which is around 10% of the spring common wheat production. The CWRS class is high protein premium bread wheat with superior milling and baking properties and is used for leavened products most commonly used in the Canadian diet. The CPSR class is multipurpose wheat with reduced protein and can be used in food, feed and industrial applications. However, compared to CWRS, CPSR wheat class yields 15-20% higher, and it was established in 1985 as a lower protein alternative to the CWRS class.

In this study, four established CWRS cultivars, two cultivars (CDC Teal and AC Superb) with a complete complement of GBSSI (A, B and D genomes), and two cultivars (AC Barrie and AC Splendor) with GBSSI-B absent (Demeke *et al*., 2000) were compared with two CPSR cultivars (AC Foremost and AC Crystal) for their starch composition and structure and their influence on *in vitro* enzymatic hydrolysis of starch in wheat grain meal and extracted starch.

## 4.3 Materials and Methods

### 4.3.1 Material

Grains from wheat (*Triticum aestivum* L), four CWRS cultivars – CDC Teal (Hughes and Hucl, 1993), AC Superb (Townley-Smith *et al*., 2010), AC Barrie (McCaig *et al*., 1996), AC Splendor (Fox *et al*., 2007) and two CPSR cultivars – AC Foremost (Thomas *et al*., 1997), AC Crystal (Fernandez *et al*., 1998) were used for biochemical analysis of grain meal and isolated starch. Four replicates of each CWRS cultivar were grown in 2010 and CPSR in 2008 in standard small plots at Kernen Crop Research Farm, University of Saskatchewan, Saskatoon, Canada (Lat: 52.2° N, Lon: 106.6° W, Elev: 510 m). The average maximum temperature ranged from 15.8 – 23.7°C in 2010 and 18.7 – 25.4°C in 2008 during the growing season (http://climate.weatheroffice.gc.ca). White bread (Wonder bread, George Weston Limited, Toronto ON, Canada) was used as control for *in vitro* hydrolysis study and calculation of hydrolysis index. Ground meal was obtained by grinding 10 g seeds through UDY cyclone mill (UDY Corporation, Fort Collins CO, USA) equipped with 0.5 mm screen.

### 4.3.2 Starch isolation and concentration determination

Starch was purified from wheat seeds using a modified method (Peng *et al*., 1999) involving cesium chloride density gradient centrifugation (Asare *et al*., 2011). Total starch

concentration was determined using the AACC approved method (AACC method 76-13.01), in which ground meal samples were hydrolyzed to dextrins and finally to D-glucose using α-amylase and amyloglucosidase respectively. Total starch concentration was determined as free glucose by measuring the absorbance at 510 nm (Hucl and Chibbar, 1996) and calculated on a percent dry weight basis (McCleary *et al*., 1997).

### 4.3.3 Protein, crude lipid and beta-glucan concentration determination

Protein concentration was determined by combustion method with the FP-528 Protein/Nitrogen Analyzer (LECO Corporation, St. Joseph MI, USA). Percent protein (%P) concentration was obtained by nitrogen (%N) quantification using the formula: %P = %N x C, where C is 5.7 for wheat (AACC method 46-30.01). Crude lipid concentration was determined by ANKOM$^{XT15}$ extractor (ANKOM Technology, Macedon NY, USA) using petroleum ether as extraction solvent (AACC method 30-25.01). Percent lipid was expressed as weight of lipid per gram dry weight of the initial material used. Beta-glucan concentration was determined using enzymatic method (AACC method 32-23.01), where ground wheat meal was digested with lichenase and β-glucosidase. Mixed linkage β-glucan concentration was calculated as free glucose by measuring absorbance at 510 nm.

### 4.3.4 Amylose concentration determination

High performance size exclusion chromatography (HPSEC) was used to determine the amylose concentration (Demeke *et al*., 1999). Starch samples were debranched using isoamylase, freeze-dried, suspended in dimethyl sulfoxide (DMSO) and injected into the column (PL gel MiniMix 250 x 4.6 mm ID column, Polymer Laboratories Inc., Amherst MA, USA) (Asare *et al*., 2011). Data was collected and analyzed using Empower 1154 chromatography software (Waters Corporation, Milford MA, USA).

### 4.3.5 Starch granule size distribution analysis

Granule size distribution (by volume) of starch slurries was determined using a laser diffraction particle size analyser (Hydro 2000S, Malvern Instruments, Malvern WR, UK) (Asare *et al*., 2011). Volume percent of particle size distribution values were obtained using Malvern Mastersizer 2000 software (Malvern Instruments, Malvern WR, UK).

### 4.3.6 Amylopectin chain length distribution analysis

Chain length distribution of amylopectin molecules was determined by fluorophore assisted capillary electrophoresis (FACE) (O'Shea *et al*., 1998) using Proteome Lab PA800 (Beckman Coulter, Fullerton CA, USA) equipped with a 488 mm laser module. A modified debranching protocol (Asare *et al*., 2011) was used to obtain unit amylopectin chains, which were labeled using 8-aminopyrene 1,2,6-triphosphate (APTS) in the presence of sodium cyano boro hydride/ tetra hydro furan (THF). The N-CHO (PVA) capillary with pre-burned window (50 μm ID, 50.2 cm total length) was used to separate debranched samples.

### 4.3.7 *In vitro* kinetics of enzymatic starch hydrolysis

Wheat and pure starch samples were enzymatically hydrolyzed *in vitro* for kinetic analysis, using a modified AACC method (AACC method 32-40.01). Meal and pure starch samples (100 mg) were incubated with pancreatic α-amylase and amyloglucosidase. For kinetic analysis of starch hydrolysis, reaction mixtures were incubated for 0, 30, 60, 90, 120 and 240 min at 37 °C. At the end of each time period a 1 mL aliquot was taken from the reaction mixture and the enzymatic reaction was terminated with an equal volume of 99% (v/v) ethanol and processed using the AACC approved method (AACC method 32-40.01). At each incubation time non-digested and digested starch concentrations were determined as free glucose using the GOPOD method (McCleary *et al*., 1997). Rate of starch digested (hydrolyzed) was expressed as total starch (%) at the end of each interval. Hydrolysis index was calculated using a non-linear model described previously (Goñi *et al*., 1997; Frei *et al*., 2003).

### 4.3.8 Statistical analyses

ANOVA of the means, multiple means comparisons using Tukey's multiple range tests at $p < 0.05$ and Person's bivariate correlations were performed with SPSS V. 19.0 software (SPSS Inc., Chicago IL, USA).

## 4.4 Results and Discussion

In a cereal grain, carbohydrates can be classified into available and non-available, based on their hydrolysis during the passage of food products in the human and animal digestive tract

(McCance and Lawrence, 1929). Available carbohydrates are broken down and absorbed by the human digestive tract to give energy to the body, while non-available carbohydrates in the large intestine produce short-chain fatty acids resulting in low usable energy. Modern lifestyle combined with major diet changes has resulted in increased rates of obesity and associated diseases (Polsky, 2012). Such problems can be reduced by consuming food products with an increased amount of non-available carbohydrates such as foods high in resistant starch.

### 4.4.1 Grain constituents in wheat cultivars

In the six wheat cultivars studied the total starch concentration was significantly ($p < 0.05$) different and ranged from 59.9% to 81.0% (Table 4.1). The two CPSR cultivars showed higher starch concentration compared to the four CWRS cultivars. AC Splendor showed the lowest amount of starch as compared to other CWRS cultivars. Amylose concentration varied between 26.5% (CDC Teal) and 30.3% (AC Crystal). Although, AC Barrie and AC Splendor have previously been reported to have null GBSSI-B (Demeke *et al*., 2000), however its absence did not affect amylose concentration (Table 4.1). In wheat grains, absence of one GBSSI isoform does not significantly change amylose concentration (Demeke *et al*., 1999). This suggests that in wheat during grain starch synthesis, genome-specific GBSSI activity is not limiting to affect amylose concentration. As expected CWRS cultivars had higher protein concentration compared to CPSR cultivars. Highest protein concentration was observed in CWRS AC Splendor (15.7%) and lowest in CPSR AC Crystal (10.6%). Lipid concentration varied significantly ($p < 0.05$) ranging from 1.1% - 2.8%. No significant ($p < 0.05$) variation in β-glucan concentration was observed between the cultivars. The grain composition is affected by environment and the CWRS and CPSR cultivars were grown during 2010 and 2008, respectively. The results could be confounded by the environmental conditions, however, the two field trials were done in the same area and the environmental conditions (light, temperature and moisture) were very similar during the two years. Compared to CWRS, the CPSR wheat kernel is larger in size and therefore accumulates more starch, and since the germ which accumulates lipids is comparatively small in size in CPSR wheat cultivars it results in reduced lipid concentration (Table 4.1).

Table 4.1 Carbohydrate and non-carbohydrate concentrations[1] of selected wheat cultivars

| Cultivar | Class/ GBSSI protein | Concentration (%) | | | | |
|---|---|---|---|---|---|---|
| | | Total Starch | Amylose | Protein | Lipid | β- Glucan |
| CDC Teal | CWRS | 68.4 ±1.0$^{c}$ | 26.5 ±0.2$^{a}$ | 13.83 ±0.02$^{c}$ | 2.76 ±0.05$^{cd}$ | 0.77 ±0.12$^{a}$ |
| AC Superb | CWRS | 67.2 ±0.5$^{bc}$ | 28.0 ±0.1$^{ab}$ | 14.25 ±0.04$^{d}$ | 2.30 ±0.07$^{bc}$ | 0.73 ±0.04$^{a}$ |
| AC Barrie | CWRS/ null B | 64.3 ±1.2$^{b}$ | 28.5 ±0.4$^{abc}$ | 14.28 ±0.03$^{d}$ | 2.82 ±0.04$^{d}$ | 0.66 ±0.10$^{a}$ |
| AC Splendor | CWRS/ null B | 59.9 ±0.6$^{a}$ | 28.1 ±0.4$^{abc}$ | 15.69 ±0.03$^{e}$ | 2.74 ±0.09$^{cd}$ | 0.51 ±0.03$^{a}$ |
| AC Foremost | CPSR | 71.9 ±0.3$^{d}$ | 28.9 ±0.6$^{bc}$ | 13.33 ±0.13$^{b}$ | 1.98 ±0.21$^{b}$ | 0.57 ±0.10$^{a}$ |
| AC Crystal | CPSR | 81.0 ±0.4$^{e}$ | 30.3 ±0.8$^{c}$ | 10.60 ±0.04$^{a}$ | 1.11 ±0.07$^{a}$ | 0.62 ±0.13$^{a}$ |

[1] Values were calculated on dry weight basis and represent the means of three biological replications and two independent observations for each replicate ± standard error. Mean values within a column followed by different superscripts are significantly different ($p < 0.05$), with superscript 'a' being the lowest value.

Pearson's correlation analysis suggests that starch concentration showed a positive correlation with amylose (r = 0.52, $p < 0.05$), while negative correlation with protein (r = -0.97, $p < 0.01$) and lipid (r = -0.88, $p < 0.01$) (Table 4.2). AC Crystal, for instance, showed significantly higher starch and amylose concentration, and lower protein and lipid concentration. A negative correlation between starch and protein has already been reported in wheat (Hucl and Chibbar, 1996).

### 4.4.2 Starch granule size distribution

On the basis of diameter, starch granules can be divided into C-type starch granules (< 5 μm), B-type starch granules (5 - 15 μm) and A-type starch granules (> 15 μm) (Bechtel *et al*., 1990). The large (A-type) and small (B- and C-type) starch granules are physiologically distinct and synthesized at different stages of grain development (Jaiswal *et al*., 2010). A-type starch granules appear four days post anthesis (DPA) and continue to increase in size, while B-type starch granules develop 12-14 DPA and remain considerably smaller. C-type starch granules start forming 21 DPA and keep accumulating throughout the grain filling period (Raeker *et al*., 1998; Rahman *et al*., 2000).

In the six wheat cultivars studied, starch granule diameter ranged from 1 μm – 60 μm. The median size for the large starch granules (A-type) was 17.4 μm for CWRS cultivars, while for CPSR cultivars it was 22.9 μm (Figure 4.1 A) suggesting a higher granule size in CPSR cultivars. Starch granule size distribution also varied significantly ($p < 0.05$) within classes. The CPSR cultivar, AC Foremost, had a significantly ($p < 0.05$) reduced volume of A-type starch granules (54.7%), consequently the volume of B-type starch granules was higher (38.0%) compared to all other cultivars (Figure 4.1 B).

### 4.4.3 Amylopectin chain length distribution

Relative normalizedarea percent versus degree of polymerization for amylopectin chain length distribution showed polymodal distribution of chains. Based on changing nodes of the distribution curve, the area was divided into seven regions with R-I (DP 6-8), R-II (DP 9-11), R-III (DP 12-14), R-IV (DP 15-18), R-V (DP 19-22), R-VI (DP 23-36) and R-VII (DP 37-45) (Figure 4.2). The average DP of amylopectin chains did not differ between the six wheat

Table 4.2 Correlation analysis between grain meal characteristics and *in vitro* enzymatic starch hydrolysis parameters

| | Starch | Amylose | Protein | Lipid | β- Glucan | RDS M | SDS M | RS M | HI M |
|---|---|---|---|---|---|---|---|---|---|
| Starch | 1 | 0.52* | -0.97** | -0.88** | 0.11 | -0.47 | 0.30 | 0.41 | -0.73** |
| Amylose | | 1 | -0.58* | -0.69** | -0.45 | -0.31 | 0.61** | 0.15 | -0.60** |
| Protein | | | 1 | 0.87** | -0.09 | 0.40 | -0.31 | -0.39 | 0.65** |
| Lipid | | | | 1 | 0.17 | 0.51* | -0.50* | -0.39 | 0.82** |
| β- Glucan | | | | | 1 | 0.23 | 0.00 | -0.42 | 0.32 |
| RDS M | | | | | | 1 | -0.26 | -0.31 | 0.65** |
| SDS M | | | | | | | 1 | -0.50* | -0.40 |
| RS M | | | | | | | | 1 | -0.49* |
| HI M | | | | | | | | | 1 |

*. Significance at $p < 0.05$. **. Significance at $p < 0.01$.

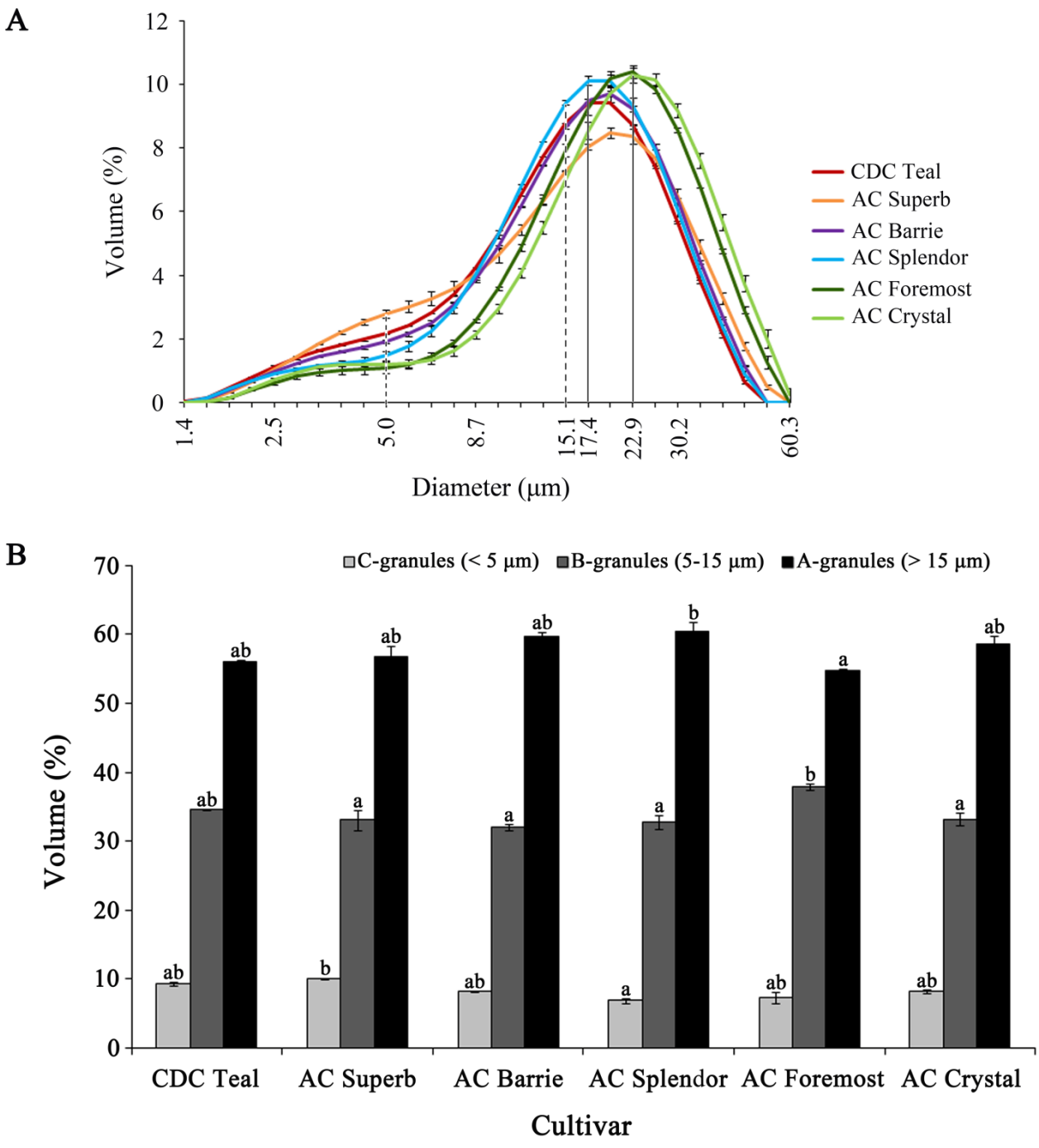



Figure 4.1 Starch granule size distribution in selected wheat cultivars

(A) Starch granule size distribution in CWRS and CPSR cultivars. Dotted lines represent different size-classes of starch granules: 0-5 µm (C-granules), 5-15 µm (B-granules) and > 15µm (A-granules). Solid lines represent median size of large granules. (B) Volume occupied by starch granules of different size ranges in CWRS and CPSR cultivars. Values are means of three replicates ± standard error. Different letters on the same colored columns indicate they are significantly different ($p < 0.05$).

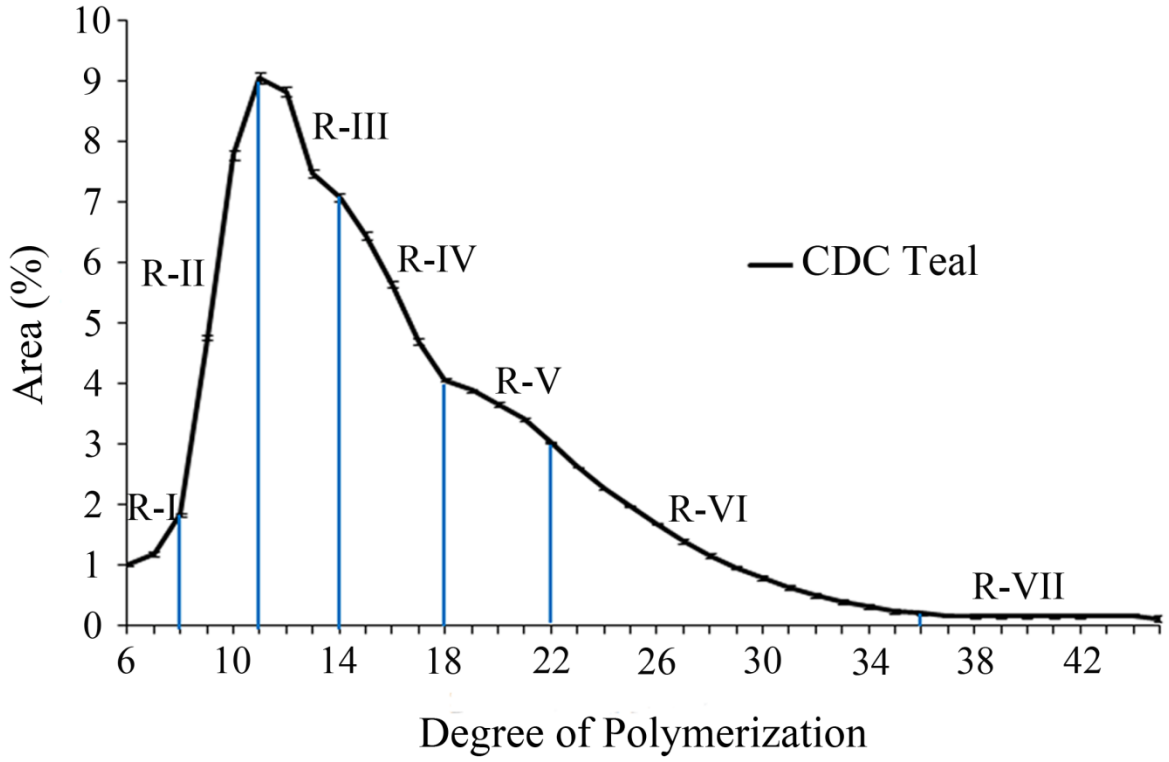



Figure 4.2 Amylopectin chain length analysis for starch extracted from CDC Teal

The amylopectin unit chains were divided into seven regions: R-I (DP 6-8), R-II (DP 9-11), R-III (DP 12-14), R-IV (DP 15-18), R-V (DP 19-22), R-VI (DP 23-36), R-VII (DP 37-45), on the basis of changing slopes of the curve.

cultivars studied (Table 4.3). However, medium sized glucan chains (DP 15-18) were significantly ($p < 0.05$) lower in the CPSR cultivars compared to the CWRS cultivars. On the other hand, longer glucan chains (DP 37-45) were higher in CPSR compared to CWRS wheat cultivars, although only AC Foremost showed significantly ($p < 0.05$) higher percentage than all other wheat cultivars (Table 4.3).

In a starch granule, amylose is present in both crystalline and amorphous regions, therefore, the organization of semi-crystalline lamellae within granules or the arrangement of amylopectin chains could be affected by amylose concentration (Blazek *et al*., 2009). In this study, a negative correlation of amylose concentration with R-IV (r = -0.69, $p < 0.01$) and R-V (r = -0.72, $p < 0.01$), and positive correlation with R-II (r = 0.57, $p < 0.05$), R-III (r = 0.55, $p < 0.05$) and R-VII (r = 0.52, $p < 0.05$) was observed (data not shown). In barley, amylose concentration is related to the amylopectin average DP (Fredriksson *et al*., 1998), however no such correlation was observed in this study.

### 4.4.4 *In vitro* starch enzymatic hydrolysis study

To study the influence of grain constituents concentration and structure on starch digestibility, *in vitro* starch hydrolysis assays were performed both with wheat grain meal and pure starch, with white bread as standard (Goñi *et al*., 1997). White bread had the highest rate of hydrolysis, with glucose released from starch reaching 90% in 30 min and the curve achieved saturation in 90 min (Figure 4.3). In wheat grain meal samples, 50% of starch hydrolysis was observed within 120 min of sample incubation with α-amylase and amyloglucosidase enzymes. As expected, hydrolysis of pure starch was faster ($t_{50}$ = 60 min) compared to meal ($t_{50}$ = 120 min).

On the basis of enzymatic hydrolysis, starch can be divided into readily digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS), which are hydrolyzed within 20-30 min, 120 min, and more than 120 min respectively, when incubated with starch hydrolytic enzymes (Sajilata *et al*., 2006). In meal, RDS values did not show significant differences among the six wheat cultivars studied (Table 4.4). SDS was lowest in CDC Teal (66.1%), and highest in AC Superb (76.4%) compared to the wheat cultivars studied. RS content was significantly ($p <$

Table 4.3 Amylopectin chain length distribution[1] in selected wheat cultivars

| Cultivar | Class/ GBSSI protein | Distribution (%) | | | | | | | Average DP |
|---|---|---|---|---|---|---|---|---|---|
| | | R-I (DP 6-8) | R-II (DP 9-11) | R-III (DP 12-14) | R-IV (DP 15-18) | R-V (DP 19-22) | R-VI (DP 23-36) | R-VII (DP 37-45) | |
| CDC Teal | CWRS | 4.00 ±0.08$^{a}$ | 21.58 ±0.20$^{a}$ | 23.37 ±0.23$^{a}$ | 20.84 ±0.19$^{b}$ | 13.96 ±0.11$^{c}$ | 14.97 ±0.41$^{a}$ | 1.28 ±0.27$^{a}$ | 16.28 ±0.14$^{a}$ |
| AC Superb | CWRS | 3.86 ±0.04$^{a}$ | 21.25 ±0.34$^{a}$ | 22.94 ±0.36$^{a}$ | 20.54 ±0.21$^{b}$ | 14.00 ±0.04$^{c}$ | 15.77 ±0.54$^{a}$ | 1.63 ±0.30$^{ab}$ | 16.52 ±0.20$^{a}$ |
| AC Barrie | CWRS/ null B | 3.97 ±0.04$^{a}$ | 21.68 ±0.09$^{a}$ | 23.07 ±0.18$^{a}$ | 20.33 ±0.13$^{b}$ | 13.84 ±0.07$^{bc}$ | 15.57 ±0.17$^{a}$ | 1.54 ±0.15$^{ab}$ | 16.43 ±0.09$^{a}$ |
| AC Splendor | CWRS/ null B | 3.68 ±0.07$^{a}$ | 21.12 ±0.25$^{a}$ | 23.26 ±0.24$^{a}$ | 20.45 ±0.21$^{b}$ | 13.94 ±0.08$^{c}$ | 16.26 ±0.41$^{a}$ | 1.29 ±0.15$^{a}$ | 16.49 ±0.16$^{a}$ |
| AC Foremost | CPSR | 3.70 ±0.10$^{a}$ | 21.62 ±0.39$^{a}$ | 23.41 ±0.36$^{a}$ | 19.62 ±0.06$^{a}$ | 13.05 ±0.20$^{ab}$ | 15.62 ±0.56$^{a}$ | 2.97 ±0.15$^{c}$ | 16.82 ±0.16$^{a}$ |
| AC Crystal | CPSR | 3.88 ±0.19$^{a}$ | 22.88 ±0.87$^{a}$ | 24.47 ±0.70$^{a}$ | 19.56 ±0.16$^{a}$ | 12.30 ±0.29$^{a}$ | 14.22 ±1.20$^{a}$ | 2.69 ±0.41$^{bc}$ | 16.44 ±0.35$^{a}$ |

[1] Values represent means of three replications. Means within the same column followed by different superscripts are significantly different ($p < 0.05$) with superscript 'a' being the lowest value. Average DP = $\Sigma$ ($DP_n$ x peak area) / $\Sigma$ $(\text{peak area})_n$.

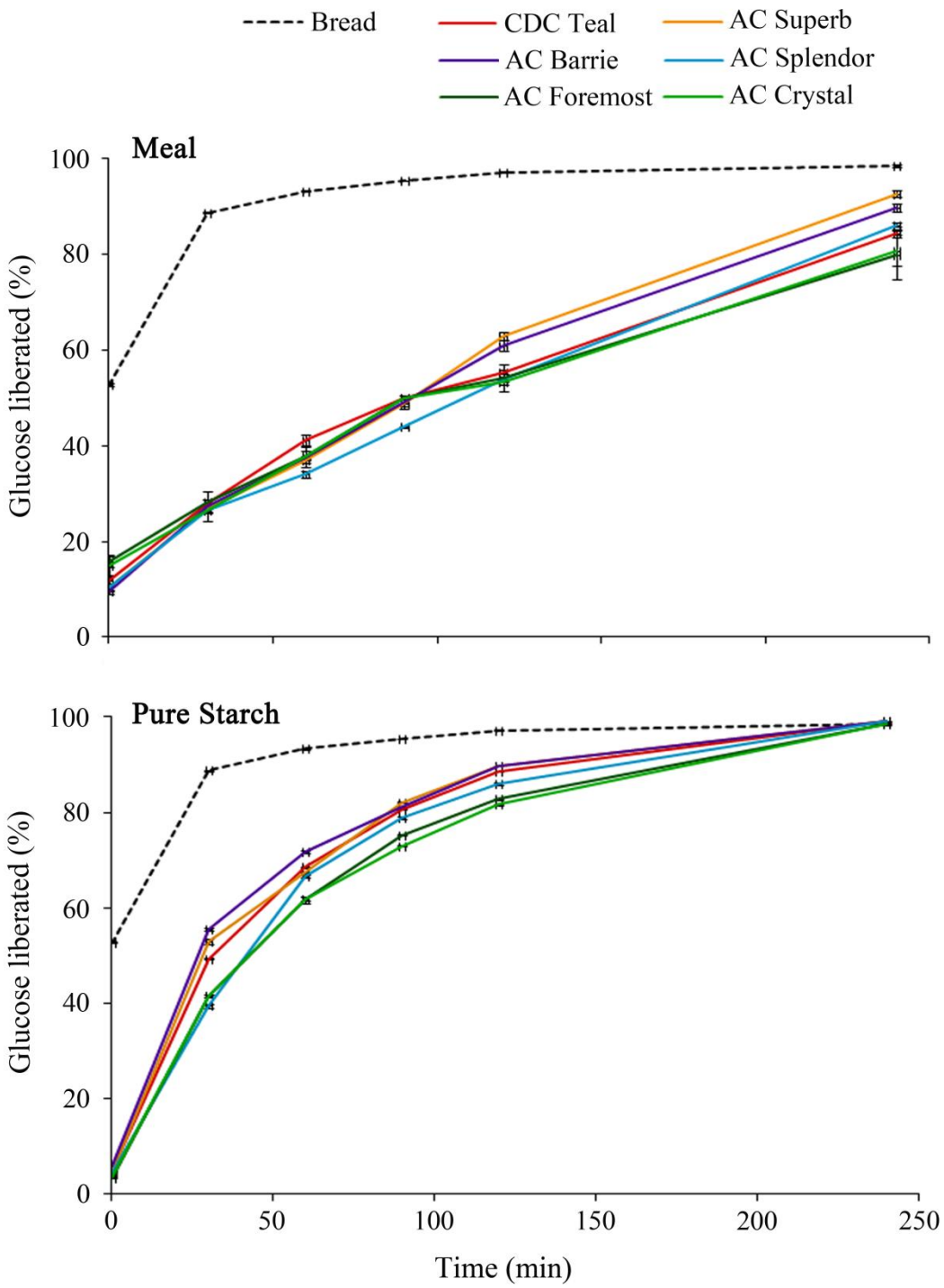



Figure 4.3 Rate of starch enzymatic hydrolysis curve showing the amount of starch hydrolyzed at selected time intervals in meal and extracted starch

Values were calculated on dry weight basis and represent the means of three biological replications and three independent observations for each replicate ± standard error.

Table 4.4 *In vitro* enzymatic hydrolysis analysis of meal and extracted starch samples[1] from selected wheat cultivars

| Cultivar | Class/ GBSSI protein | RDS M | RDS PS | SDS M | SDS PS | RS M | RS PS | HI M | HI PS |
|---|---|---|---|---|---|---|---|---|---|
| CDC Teal | CWRS | 16.0 ±1.7$^{a}$ | 44.3 ±0.1$^{c}$ | 66.1 ±0.7$^{a}$ | 54.8 ±0.1$^{c}$ | 15.6 ±0.9$^{b}$ | 0.9 ±0.0$^{b}$ | 61.3 ±0.6$^{bc}$ | 139.4 ±0.3$^{d}$ |
| AC Superb | CWRS | 16.4 ±1.1$^{a}$ | 48.3 ±0.3$^{d}$ | 76.4 ±0.9$^{c}$ | 50.9 ±0.3$^{b}$ | 7.3 ±0.8$^{a}$ | 0.8 ±0.0$^{a}$ | 56.6 ±0.9$^{b}$ | 139.1 ±0.2$^{d}$ |
| AC Barrie | CWRS/ null B | 17.9 ±0.4$^{a}$ | 49.6 ±0.2$^{d}$ | 71.9 ±1.3$^{bc}$ | 49.4 ±0.2$^{a}$ | 10.2 ±0.8$^{ab}$ | 0.9 ±0.0$^{b}$ | 64.3 ±0.2$^{c}$ | 139.7 ±0.2$^{d}$ |
| AC Splendor | CWRS/ null B | 15.9 ±1.2$^{a}$ | 34.9 ±0.2$^{a}$ | 70.3 ±1.4$^{ab}$ | 64.0 ±0.2$^{e}$ | 13.8 ±0.4$^{b}$ | 1.0 ±0.0$^{b}$ | 57.2 ±0.1$^{b}$ | 126.0 ±0.8$^{c}$ |
| AC Foremost | CPSR | 13.6 ±0.2$^{a}$ | 38.1 ±0.4$^{b}$ | 73.1 ±1.2$^{bc}$ | 60.6 ±0.4$^{d}$ | 15.4 ±2.2$^{b}$ | 1.4 ±0.0$^{c}$ | 37.9 ±1.9$^{a}$ | 119.0 ±0.4$^{b}$ |
| AC Crystal | CPSR | 14.2 ±1.1$^{a}$ | 37.8 ±0.4$^{b}$ | 74.5 ±0.1$^{bc}$ | 60.9 ±0.4$^{d}$ | 16.3 ±1.8$^{b}$ | 1.4 ±0.0$^{c}$ | 40.6 ±1.4$^{a}$ | 116.4 ±0.7$^{a}$ |

[1] Values are based on means of three biological replicates with two independent observations for each replicate ± standard error. Mean values within a column followed by different superscripts are significantly different ($p < 0.05$), with superscript 'a' being the lowest value. M stands for Meal and PS stands for Pure Starch.

*0.05*) lower in AC Superb (7.3%). In pure starch, RDS, SDS and RS values showed considerable variation between the wheat cultivars. RDS concentration ranged from 34.9% (AC Splendor) to 49.6% (AC Barrie). SDS concentration was highest in AC Splendor (64.0%) and lowest in AC Barrie (49.4%). AC Superb showed the lowest RS concentration (0.8%), while AC Foremost and AC Crystal showed significantly ($p < 0.05$) higher RS content.

Hydrolysis index (HI) is a mathematical predictor of glycemic index (GI), the postprandial blood glucose response (Goñi *et al*., 1997). HI was calculated from a first order kinetic equation, with white bread as standard (Goñi *et al*., 1997; Frei *et al*., 2003). HI in both meal and pure starch showed considerable differences among the wheat cultivars (Table 4.4). In the two CPSR wheat cultivars both grain meal and pure starch showed lower HI compared to grain meal and starch in the CWRS cultivars. HI for meal was lowest in AC Foremost (37.9), while HI for pure starch was lowest in AC Crystal (116.4). Among the CWRS cultivars, significantly ($p < 0.05$) lower HI was observed in AC Splendor than other wheat cultivars studied. Overall, CPSR cultivars have reduced digestibility as compared to CWRS, due to lower HI and higher RS concentration.

### 4.4.5 Starch digestibility in relation with starch components

Various inherent properties of starch have been implicated to affect starch hydrolysis in different plants (Oates, 1997; Themeier *et al*., 2005; Sang *et al*., 2008). Amylose concentration affects starch hydrolysis (Sajilata *et al*., 2006). In rice, *in vitro* enzymatic digestibility studies have shown a linear increase in RS concentration with increasing amylose concentration (Frei *et al*., 2003; Chung *et al*., 2010; Zhu *et al*., 2011). Studies in maize have also shown a positive correlation between amylose and RS content (Zhang *et al*., 2008). High amylose corn starch has been shown to result in high RS levels in mice (Ito *et al*., 1999). *In vitro* enzymatic hydrolysis study in barley (Asare *et al*., 2011) has shown that RS concentration follows the order increased amylose > non-waxy > waxy genotypes. A commercial high-amylose barley cultivar, Himalaya 292 has been developed and upon feeding to animals and humans, has shown the formation of increased RS contents (Topping *et al*., 2003). Although contrasting differences in amylose concentrations were not observed, however, a CPSR cultivar, AC Crystal showed significantly ($p < 0.05$) higher amylose concentration than other cultivars (Table 4.1). In addition, AC Crystal showed lower hydrolysis index as compared to other cultivars (Table 4.4). Both in meal and pure

starch, HI was negatively correlated with starch (r = -0.57, $p < 0.05$) and amylose (r = -0.63, $p < 0.01$) (Table 4.5). A simple explanation could be that higher concentration of starch takes more time to hydrolyze. In the case of amylose it is possible that linear chains of amylose fold in a manner that limits the access of amylases, leading to slower rate of hydrolysis. HI meal was also significantly influenced by lipid and protein content (Sajilata *et al*., 2006; Lehmann and Robin, 2007).

Amylopectin chain length distribution also affects starch digestibility (Srichuwong *et al*., 2005). In maize starch a high proportion of either very short chains or very long chains were associated with a higher SDS content, thus proposing a parabolic relationship between SDS and amylopectin fine structure (Zhang *et al*., 2008). Similarly in barley, RS concentration was shown to be negatively correlated with short chains of amylopectin (Asare *et al*., 2011). The relationship between amylopectin structure and starch digestibility has been extensively studied in rice, where longer branches have been associated with lower starch digestibility (Okuda *et al*., 2005; Benmoussa *et al*., 2007). A recent digestibility study on cooked rice grains has also suggested that a slower digestion rate was observed in starches with higher ratios of long to short amylopectin branches (Syahariza *et al*., 2013). However, rice mutants high in RS showed increased proportion of short chains with DP ≤ 12, decreased proportion of intermediate chains of DP 13-36 and a reduction in long chains with DP ≥ 37 (Shu *et al*., 2007).

Correlation analyses between amylopectin chain length distribution and starch hydrolysis parameters revealed a positive correlation of RS with R-II, RIII (DP 9-14, r = 0.55, 0.60, $p < 0.05$) and R-VII (DP 37-45, r = 0.75, $p < 0.01$); and negative correlation with R-IV, R-V (DP 15-22, r = -0.85, -0.86 $p < 0.01$) (Table 4.5). We observed that CPSR cultivars, with significantly ($p < 0.05$) higher RS than CWRS cultivars (Table 4.4), showed increased proportion of long chains of DP 37-45 and decreased chains of DP 15-22 (Table 4.3). Thus, the CPSR cultivars with lower digestibility and higher proportion of long chains concur with the parabolic relationship between slowly digestible starch and amylopectin chain length distribution model in maize (Zhang *et al*., 2008). In addition, reduced mid-length chains of amylopectin in rice have been associated with higher RS (Shu *et al*., 2007). Similarly, reduced proportion of DP 15-18 in the CPSR cultivars also supports the report in rice. Therefore, we suggest that these fractions of chains (reduced DP 15-18 and increased DP 37-45) might be contributing towards reduced rate of *in vitro* starch enzymatic hydrolysis in CPSR cultivars.

Table 4.5 Correlation analysis between grain starch characteristics and *in vitro* enzymatic starch hydrolysis parameters

| | RDS PS | SDS PS | RS PS | HI PS |
|---|---|---|---|---|
| Starch | -0.21 | 0.19 | 0.70** | -0.57* |
| Amylose | -0.30 | 0.29 | 0.64** | -0.63** |
| R-I | 0.35 | -0.36 | -0.05 | 0.19 |
| R-II | -0.21 | 0.19 | 0.55* | -0.47 |
| R-III | -0.41 | 0.39 | 0.60** | -0.58* |
| R-IV | 0.45 | -0.42 | -0.85** | 0.79** |
| R-V | 0.47 | -0.44 | -0.86** | 0.80** |
| R-VI | 0.13 | -0.11 | -0.43 | 0.34 |
| R-VII | -0.32 | 0.30 | 0.75** | -0.65** |
| Avg DP | -0.06 | 0.06 | 0.06 | -0.07 |
| C-granules | 0.66** | -0.66** | -0.49* | 0.60** |
| B-granules | -0.30 | 0.29 | 0.47 | -0.38 |
| A-granules | -0.08 | 0.09 | -0.17 | 0.03 |

*. Significance at $p < 0.05$. **. Significance at $p < 0.01$.

Although parabolic relationship between amylopectin chain length distribution and starch digestibility was evident in CPSR cultivars, however no such relation was observed in CWRS cultivars. Therefore, other factors besides amylose concentration and amylopectin chain length distribution might also influence the rate of *in vitro* starch hydrolysis. Among the CWRS cultivars, AC Splendor showed the lowest amount of RDS (34.9%), highest SDS (64.0%) and lower HI (126.0) compared to other CWRS cultivars (Table 4.4),without significant ($p < 0.05$) differences in amylose concentration or amylopectin chain length distribution, but variable starch granule size distribution (Figure 4.1). A recent study showed that wheat starch granules of < 10 μm had significantly higher rate of reducing sugar release than those > 10 μm (Teo and Small, 2012). Another study in wheat showed that small starch granules (< 10 μm) were digested to a greater extent than large (> 10 μm) starch granules initially, but after four hours, large granules were digested more extensively than small, suggesting different digestion behaviours of starch granules with different sizes (Salman *et al*., 2009). *In vitro* hydrolysis study in barley (Asare *et al*., 2011) has suggested a negative correlation between C-type starch granules and SDS. Similarly, in the present study, C-type starch granules were correlated positively ($p < 0.01$) with RDS and HI, and negatively correlated with SDS and RS in extracted starch (Table 4.5). This could be that small starch granules provide more accessible surface area for amylases to act, hence increasing starch digestibility. For instance, among the CWRS cultivars, AC Splendor with lowest HI (126.0) showed significantly ($p < 0.05$) reduced volume of C-type starch granules (~25%), and increased volume of A-type starch granules (~5%) which could be responsible for lower rate of *in vitro* starch hydrolysis.

## 4.5 Conclusions

In conclusion, the two market classes, CWRS and CPSR differ in grain composition, starch composition and structure. In addition to differences in starch and amylose concentrations in these cultivars (Hucl and Chibbar, 1996), amylopectin chain length distribution and starch granule size distribution also varies between the two classes. CPSR cultivars with increased amylopectin chains of DP 37-45, reduced chains of DP 15-18, lower volume of small C-type starch granules, showed subtle effects on reducing the *in vitro* starch hydrolysis rate. The wheat cultivars show differences in grain starch composition and structure which can have an impact on their end-use.

# 5. GENOME SPECIFIC GBSSI INFLUENCES STARCH BIOCHEMICAL AND FUNCTIONAL CHARACTERISTICS IN NEAR-ISOGENIC WHEAT (*Triticum aestivum* L.) LINES

**Study 2**[2]

Study 1 was based on wild-type non-waxy wheat cultivars, with full complement of starch synthesizing enzymes. Study 2 was conducted on near-isogenic waxy-null wheat lines with GBSSI isoproteins absent in one, two or three genomes. Similar methodologies were used to analyze various grain and starch components influencing *in vitro* starch hydrolysis potential. It was concluded that genome-specific GBSSI had subtle effects on starch composition and structure, and only the complete absence of GBSSI majorly affects starch hydrolysis. This study was a follow up to determine subtractive effects of GBSSI as opposed to the non-waxy wheats used in Study 1.

[2] **Ahuja, G.**, Jaiswal, S., Hucl, P., & Chibbar, R.N. (2013). Genome-Specific Granule-bound starch synthase I (GBSSI) Influences Starch Biochemical and Functional Characteristics in Near-Isogenic Wheat (*Triticum aestivum* L.) Lines. *Journal of Agricultural and Food Chemistry* (dx.doi.org/10.1021/jf4040767 | J. Agric. Food Chem).

## 5.1 Abstract

Near-isogenic wheat (*Triticum aestivum* L.) lines differing at the *Waxy* locus were studied for the influence of genome specific granule-bound starch synthase I (GBSSI/ Waxy, Wx-A, Wx-B, Wx-D) on starch composition, structure and *in vitro* starch enzymatic hydrolysis. Grain composition, amylose concentration, amylopectin unit-chain length distribution and starch granule size distribution varied with the loss of functional GBSSI. Amylose concentration was more severely affected in genotypes with GBSSI missing from two genomes (double nulls) than from one genome (single nulls). Unit glucan chains (DP 6-8) of amylopectin were reduced with the complete loss of GBSSI as compared to wheat starch with full complement of GBSSI. Wx-A and Wx-B had an additive effect towards short chain phenotype of waxy amylopectin. Loss of Wx-D isoprotein alone significantly ($p < 0.05$) reduced the C-type starch granules. However, absence of Wx-D in combination with Wx-A or Wx-B increased the B-type and C-type starch granules but decreased the volume of A-type starch granules. Rate of *in vitro* starch enzymatic hydrolysis was highest in completely waxy grain meal and purified starch. However, presence of Wx-D reduced wheat starch hydrolysis as it increased the large A-type starch granule content (volume %) and reduced short chains (DP 6-8) in amylopectin. Factors such as small C-type starch granules, amylose concentration and long chains of amylopectin (DP 23-45) also influenced wheat starch hydrolysis.

## 5.2 Introduction

Endosperm of a wheat grain is occupied mostly (~70%) by starch, which is a glucan homo-polymer, composed of one-quarter amylose ($10^5$–$10^6$ Da) and three-quarters amylopectin ($10^7$–$10^9$ Da). Amylose is a pre-dominantly linear molecule made up of α-1,4 linked glucose residues with a degree of polymerization of ~800 in wheat and few branches (Chibbar *et al*., 2007). Amylopectin, on the other hand, is a highly branched glucan polymer with 4-5% α-1,6 branches and degree of polymerization (DP) of $10^5$–$10^7$. The amylopectin branches form an organized structure and are categorized into: short A-chains (DP 6-12), intermediate B-chains (DP 13-24 up to 50) and a long inter-cluster C-chain, with one free reducing end per amylopectin molecule (Manners, 1989).

Amylose and amylopectin have different structural and physiological characteristics and hence exhibit different reactions within the body during digestion and subsequent release of glucose molecules for absorption (Sajilata *et al*., 2006). Various studies have shown that amylose to amylopectin ratio, degree of polymerization and/or branching of glucan polymers are important determinants of the extent of starch enzymatic hydrolysis and hence digestibility of food. Rate of starch enzymatic hydrolysis is reduced in starches with higher amylose concentration (Regina *et al*., 2006). Relation between amylopectin chain length distribution and resistant starch has also been studied in different plant systems (Srichuwong *et al*., 2005). Changes in the amylopectin chain length distribution facilitate retrogradation to produce B- and V-type crystalline structures, leading to more resistant starch. It is generally believed that increased proportion of longer chains makes the starch more resistant to digestion (Jia *et al*., 2007). A possible reason could be that longer chains form longer helices, which are further stabilized by hydrogen bonds, distributed over the entire crystalline region, hence decreasing digestibility (Lehmann and Robin, 2007).

Starch, on the basis of its digestion behaviour after consumption, can be divided into digestible and resistant starch (Berry, 1986). The portion of starch which gets digested within 20-30 min of ingestion is called Readily Digestible Starch (RDS), and that which gets digested within 120 min of ingestion is called Slowly Digestible Starch (SDS). Resistant starch (RS) is referred to that portion of starch which resists digestion after 120 min (Englyst *et al*., 1992); escapes digestion in the small intestine/ upper gastrointestinal tract and is fermented in the large intestine by gut microflora. RS offers many positive physiological effects on human body such as reduction in glycemic index, production of short chain fatty acids, particularly butyrate, which prevents colorectal cancer; and better absorption of minerals like calcium and iron (Topping and Clifton, 2001).

Amylopectin synthesis is a complex process and involves an array of enzymes. These include ADP-glucose pyrophosphorylase (AGPase), starch synthases (SSI, SSII, SSIII, and SSIV), starch branching enzymes (SBEI, SBEII) and starch debranching enzymes (DBE) (Tetlow, 2011). Amylose synthesis and elongation, however, involves a single enzyme, Granule-bound starch synthase I (GBSSI), also known as 'Waxy' protein (Tetlow, 2011). Plants lacking the *waxy* gene, which encodes GBSSI, produce starch without amylose, known as waxy starch.

GBSSI has also been reported to be involved in the elongation of amylopectin chains, particularly for very long branches (Ral *et al*., 2006).

Wheat is an allohexaploid crop (*Triticum aestivum* L., 2n = 6x = 42; BBAADD), consisting of three genomes derived from three diploid parents. Therefore, wheat endosperm has three isoforms of GBSSI encoded by the *Waxy* (*Wx*) loci; *Wx-A1*, *Wx-B1*, *Wx-D1*, located on chromosomes 7AS, 4AL (translocated from 7BS) and 7DS respectively (Chao, 1989). Absence of GBSSI in any of the three genomes would affect the concentration of amylose and amylopectin, starch structure and functional properties. The objective of this work was to study the effect of GBSSI from the three wheat genomes on starch composition, structure and *in vitro* starch hydrolysis. The terms 'GBSSI' and 'Waxy' have been used interchangeably.

## 5.3 Materials and Methods

### 5.3.1 Material

Plant material for this study included thirty-two Canadian western red spring (CWRS) near-isogenic waxy wheat (*Triticum aestivum* L.) genotypes, including four lines of each Wx-$A^+B^+D^+$, $A^-B^+D^+$, $A^+B^-D^+$, $A^+B^+D^-$, $A^-B^-D^+$, $A^-B^+D^-$, $A^+B^-D^-$ and $A^-B^-D^-$. These lines were derived by crossing CDC Teal (complete complement of *Wx* alleles; recurrent parent) with CDC Wx2 (*Wx-AIb*, *Wx-BIb*, *Wx-DIb* null alleles; donor parent); CDC Teal*7 / CDC Wx2. Both the parents were developed at University of Saskatchewan (Hughes and Hucl, 1993; Matus-Cadiz, 1999). Four replicates of each of the near-isogenic line were grown in a Randomized Complete Block Design in 2010 in Kernen (52°09' N, 106°33' W) and Goodale (52°03' N, 106°29' W) Crop Research Farms, University of Saskatchewan, Saskatoon, Canada. One thousand seeds from each genotype were weighed for thousand grain weight (TGW) determination. To prepare grain meal, 10 g of seeds were ground by UDY mill (UDY Corporation, Fort Collins, CO) equipped with a 0.5 mm sieve. White bread (Wonder bread, George Weston Limited, Toronto ON, Canada) was used as control for *in vitro* hydrolysis study and calculation of hydrolysis index.

### 5.3.2 Analysis of GBSSI polypeptides

Starch was purified from wheat seeds (7-10) using a modified method involving cesium chloride density gradient centrifugation (Peng *et al*., 1999). Proteins were extracted from the starch granules by suspending 10 mg starch in 300 µL denaturing electrophoresis buffer (Demeke *et al*., 1997). GBSSI was separated by polyacrylamide (10% v/v, 1 mm thick gel) electrophoresis (Protean II, Biorad, Hercules CA, USA) under denaturing conditions (Demeke *et al*., 1997). Separated polypeptides were visualized by silver staining (Gromova and Celis, 2006). The gel-electrophoresis separated polypeptides were transferred on a nitrocellulose membrane at 10 V for 4 h (Gao and Chibbar, 2000). Membrane with transferred polypeptides was processed and incubated with primary antibodies raised against wheat GBSSI (Demeke *et al*., 1999). The antigen-antibody complex was detected with goat anti-rabbit alkaline phosphate conjugate using BCIP/NBT immunoscreening colour development kit (Biorad, Hercules CA, USA).

### 5.3.3 Grain constituents concentration determination

Total starch concentration was determined using the AACC approved method (AACC method 76-13.01), where ground meal samples (100 mg in duplicate) were hydrolyzed to dextrins and further to D-glucose using α-amylase and amyloglucosidase respectively. Total starch concentration was determined as free glucose by determining the absorbance at 510 nm (Hucl and Chibbar, 1996) and calculated on a percent dry weight basis (McCleary *et al*., 1997). Protein concentration was determined by combustion method with the FP-528 Protein/Nitrogen Analyzer (LECO Corporation, St. Joseph MI, USA). Percent protein concentration was obtained by the formula: %P = %N x C, where C is 5.7 for wheat (AACC method 46-30.01). Crude lipid concentration was determined by ANKOM$^{XT15}$ extractor using petroleum ether as extraction solvent (AACC method 30-25.01). Percent lipid was expressed as weight of lipid per gram dry weight of the initial material used. Beta-glucan concentration was determined using enzymatic method (AACC method 32-23.01). Ground wheat meal (100 mg in duplicate) was digested with lichenase and β-glucosidase. Mixed linkage β-glucan concentration was calculated as free glucose by determining absorbance at 510 nm (McCleary and Codd, 1991).

### 5.3.4 Amylose concentration determination

High performance size exclusion chromatography (HPSEC) was used to determine the amylose concentration as described (Demeke *et al*., 1999). Starch samples were debranched using isoamylase, freeze-dried, suspended in dimethyl sulfoxide (DMSO), and injected (40 µL) into a column (PL gel MiniMix 250 x 4.6 mm ID column, Polymer Laboratories Inc., Amherst MA, USA). Amylose and amylopectin were separated using HP-SEC (Waters 600 Controller, Waters 610 Fluid Unit, Waters 717 plus Autosampler, Waters 410 Differential Refractometer, Waters Corporation, Milford, MA). Data was collected and analyzed using Empower 1154 chromatography software (Waters Corporation, Milford MA, USA).

### 5.3.5 Amylopectin chain length distribution analysis

Glucan chain length distribution of amylopectin molecules was determined by fluorophore assisted capillary electrophoresis (FACE) (O'Shea *et al*., 1998) using Proteome Lab PA800 (Beckman Coulter, Fullerton CA, USA) equipped with a 488 mm laser module. A modified starch debranching protocol (Asare *et al*., 2011) was used to obtain unit amylopectin chains, which were labeled using 8-aminopyrene 1, 2, 6-trisulfonate (APTS) in the presence of sodium cyanoborohydride/ tetrahydrofuran. The N-CHO (PVA) capillary with pre-burned window (50 µm ID, 50.2 cm total length) was used to separate debranched samples.

### 5.3.6 Starch granule size distribution analysis

Starch granule size distribution (by volume) in starch slurries was determined using a laser diffraction particle size analyser (Hydro 2000S, Malvern Instruments, Malvern WR, UK) (Asare *et al*., 2011). Volume percent particle size distribution values were obtained using Malvern Mastersizer 2000 software (Malvern Instruments, Malvern WR, UK).

### 5.3.7 *In vitro* kinetics of enzymatic starch hydrolysis

Wheat grain meal and pure starch samples were enzymatically hydrolyzed *in vitro* for kinetic analysis, following a modified AACC approved method (AACC method 32-40.01). Meal and pure starch samples (100 mg) were mixed with pancreatic α-amylase (10 mg/mL) and amyloglucosidase (3 U/mL, Megazyme International Ltd., Wicklow, Ireland) for starch

hydrolysis. Reaction mixtures were incubated for 0, 30, 60, 90, 120 and 240 min at 37 °C, during which starch was hydrolyzed to D-glucose. A 1 mL aliquot was taken from the reaction mixture after every incubation time and reaction was terminated by adding an equal volume of 99% (v/v) ethanol and processed following the AACC approved method with modifications. Resistant starch and soluble starch concentrations were measured as free glucose by determining its absorbance at 510 nm (McCleary *et al*., 1997). Rate of starch hydrolyzed was expressed as percent of total starch at the end of each interval. Hydrolysis index was calculated using a non-linear model described previously (Goñi *et al*., 1997; Frei *et al*., 2003).

### 5.3.8 Statistical analyses

Analysis of variation (ANOVA) of the means, multiple means comparisons using Tukey's multiple range tests at $p < 0.05$ and Pearson's bivariate correlations were performed with SPSS V. 19.0 software (SPSS Inc., Chicago IL, USA). Dendrograms were obtained by Minitab V. 16.0 software (Minitab Inc., State College PA, USA).

## 5.4 Results

### 5.4.1 Screening of wheat genotypes

Gel electrophoresis analysis of starch granule proteins showed that the most prominent polypeptide was GBSSI (mol. wt. ~60 kDa), which showed three bands, one originating from each genome (Figure 5.1). The waxy A, D and B isoproteins have apparent molecular masses of 62.7, 58.7 and 56.7 kDa respectively (Zhao and Sharp, 1996). Although GBSSI-A was clearly distinct, however GBSSI-B and -D were difficult to separate owing to their similar relative mobility on denaturing polyacrylamide gel electrophoresis, which has also been observed previously (Demeke *et al*., 1997). Three resolved polypeptides were observed at 100-115 kDa which could be SSII, as previously identified by immunoblotting antibodies against recombinant SSIIa-1 (Gao and Chibbar, 2000). In wheat, mature SBEI has been predicted to have a molecular mass of 87 kDa (Repellin *et al*., 2001), which was also observed in the present study. It has also been shown that *SBEI* gene produces alternatively spliced transcripts, which gives an 87.4 kDa mature protein (Båga *et al*., 1999). A larger variant of starch branching enzyme, SBEIc (152 kDa) (Båga *et al*., 2000), however was not observed. At ~77 kDa, a polypeptide was observed in

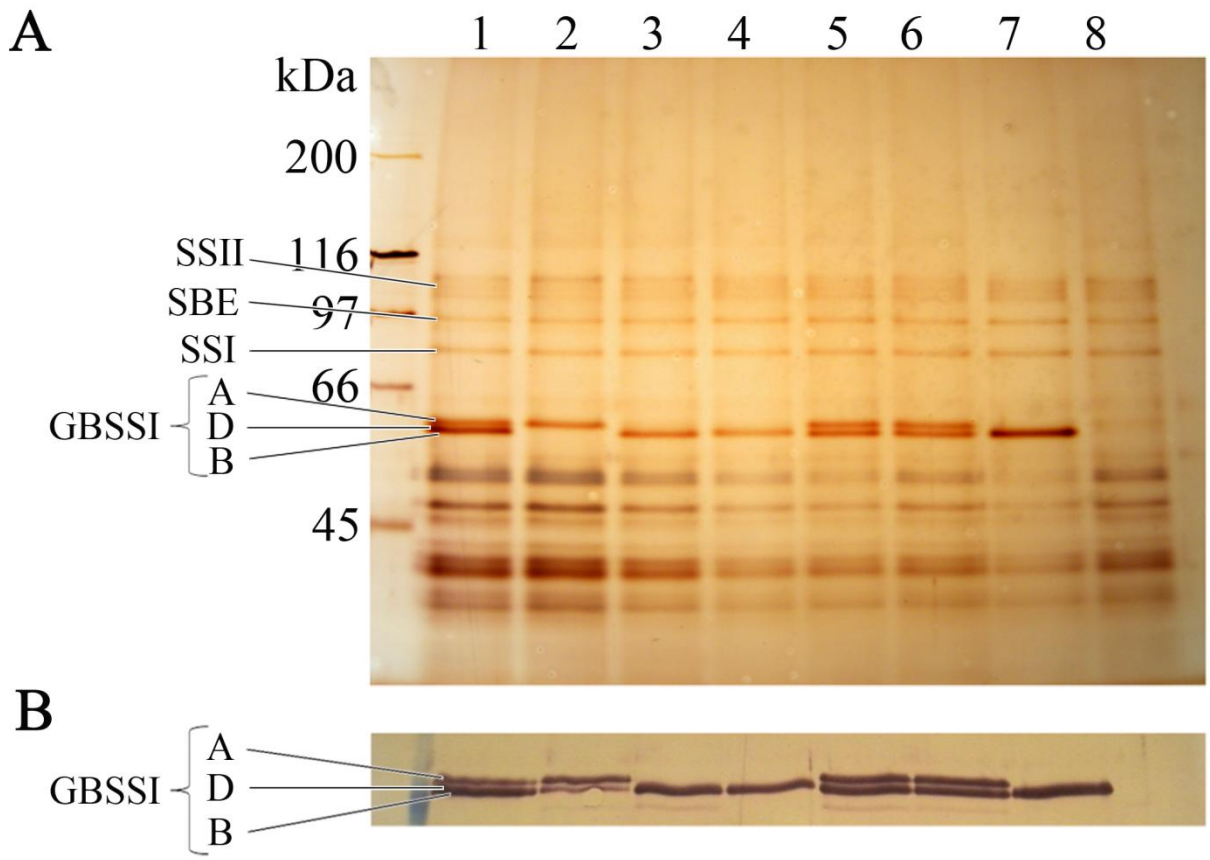



Figure 5.1 Analysis of GBSSI accumulation in wheat endosperm starch granules

(A) Denaturing polyarcylamide gel electrophoresis of starch granule-bound proteins. Lane 1: Wx-$A^{+}B^{+}D^{+}$, Lane 2: Wx-$A^{+}B^{-}D^{-}$, Lane 3: Wx-$A^{-}B^{+}D^{-}$, Lane 4: Wx-$A^{-}B^{-}D^{+}$, Lane 5: Wx-$A^{+}B^{+}D^{-}$, Lane 6: Wx-$A^{+}B^{-}D^{+}$, Lane 7: Wx-$A^{-}B^{+}D^{+}$, Lane 8: Wx-$A^{-}B^{-}D^{-}$. Molecular mass of protein standards and migration of known starch granule-proteins is shown on the left. (B) Immuno-reactive signal obtained with GBSSI - specific antibodies.

all genotypes which could be starch synthase I (SSI) (Peng *et al*., 2001). In wheat the N- terminal sequence of 75 kDa polypeptide shows homology to rice soluble starch synthase (Rahman *et al*., 1995). In addition, various other lower molecular weight proteins such as puroindolines, 15 kDa (Gautier *et al*., 1994) were observed, which showed some differences between genotypes.

### 5.4.2 Carbohydrates concentration

Thousand grain weight (TGW) in Wx-$A^+B^+D^+$ genotype was 40.1 g, while it varied significantly ($p < 0.05$) between 39.3 – 43.6 g for the other waxy-null genotypes (Table 5.1). Lowest grain weight (39.3 g) was observed for Wx-$A^-B^-D^-$ and Wx-$A^-D^-$ genotypes, whereas highest grain weight was observed for Wx-$D^-$ genotype (43.6 g).

The total starch concentration in Wx-$A^+B^+D^+$ was 63.1%, while it ranged from 57.7 to 64.8% in the partial and completely waxy genotypes (Table 5.1). Wx-$A^-B^-D^-$ showed significantly ($p < 0.05$) lower amount of starch (58.1%). However, double null genotypes showed a higher starch concentration (62.9 – 64.8%) than single null genotypes (57.7 – 60.5%). Since starch content is positively correlated with grain yield (Hucl and Chibbar, 1996), increase in TGW should be related to higher total starch concentration. However, Wx-$D^-$ genotype with higher TGW, had lower starch concentration (57.7%).

Amylose concentration varied significantly ($p < 0.05$) among the different waxy-null genotypes. Wx-$A^+B^+D^+$ genotype showed a higher amylose concentration of 28.7%, while the Wx-$A^-B^-D^-$ genotype showed non-detectable to very low (2.7%) concentration (Table 5.1). Among the partial waxy genotypes, amylose concentration varied from 21.5 – 23.1% in double nulls, whereas it varied from 26.8 – 28.3% in single null genotypes, concurring with a previous report that double nulls are more affected than single nulls (Demeke *et al*., 1999). This suggests that genome specific GBSSI has substantial dosage effect on amylose concentration. However, since the amylose concentration was not completely reduced in single and double null genotypes, it can be suggested that genome specific GBSSI activity is not limiting for amylose synthesis, and shows a compensatory role of Wx proteins from the different genomes.

Table 5.1 Proximate analysis[1] of wheat grains with different combinations of waxy (GBSSI) isoproteins

| Genome Composition | Thousand Grain Weight (g) | Concentration (%) | | | | |
|---|---|---|---|---|---|---|
| | | Total Starch | Amylose | Protein | Lipid | β-Glucan |
| $A^+B^+D^+$ | 40.1 ±0.4$^{ab}$ | 63.1 ±0.5$^{bc}$ | 28.7 ±0.5$^{c}$ | 15.86 ±0.07$^{cd}$ | 2.32 ±0.01$^{ab}$ | 0.73 ±0.01$^{a}$ |
| $A^-B^+D^+$ | 40.9 ±0.7$^{abc}$ | 59.6 ±0.5$^{ab}$ | 26.8 ±0.4$^{c}$ | 15.54 ±0.19$^{cd}$ | 2.33 ±0.03$^{ab}$ | 0.73 ±0.01$^{a}$ |
| $A^+B^-D^+$ | 42.2 ±0.1$^{bc}$ | 60.5 ±0.5$^{ab}$ | 28.3 ±0.6$^{c}$ | 16.70 ±0.75$^{d}$ | 2.37 ±0.05$^{ab}$ | 0.70 ±0.02$^{a}$ |
| $A^+B^+D^-$ | 43.6 ±0.5$^{c}$ | 57.7 ±0.1$^{a}$ | 27.0 ±0.4$^{c}$ | 18.84 ±0.02$^{e}$ | 2.31 ±0.02$^{ab}$ | 0.66 ±0.01$^{a}$ |
| $A^-B^-D^+$ | 41.0 ±0.9$^{abc}$ | 62.9 ±1.1$^{bc}$ | 23.1 ±0.4$^{b}$ | 14.65 ±0.24$^{bc}$ | 2.33 ±0.03$^{ab}$ | 0.86 ±0.01$^{b}$ |
| $A^-B^+D^-$ | 39.3 ±0.4$^{a}$ | 63.2 ±0.6$^{bc}$ | 22.5 ±0.2$^{b}$ | 12.95 ±0.04$^{a}$ | 2.25 ±0.02$^{a}$ | 0.88 ±0.01$^{b}$ |
| $A^+B^-D^-$ | 40.8 ±0.6$^{ab}$ | 64.8 ±0.9$^{c}$ | 21.5 ±0.4$^{b}$ | 13.68 ±0.17$^{ab}$ | 2.49 ±0.06$^{b}$ | 0.86 ±0.02$^{b}$ |
| $A^-B^-D^-$ | 39.3 ±0.3$^{a}$ | 58.1 ±0.3$^{a}$ | 2.7 ±0.2$^{a}$ | 15.59 ±0.02$^{cd}$ | 2.81 ±0.06$^{c}$ | 1.08 ±0.01$^{c}$ |

[1] Values were calculated on dry weight basis (except amylose is percent of total starch) and represent the means of three biological replications and two independent observations for each replicate ± standard error. Mean values within a column followed by different superscripts are significantly different ($p < 0.05$), with superscript 'a' representing the smallest value.

### 5.4.3 Protein concentration

Protein concentration in Wx-$A^+B^+D^+$ and Wx-$A^-B^-D^-$ was 15.9% and 15.6% respectively, while it ranged from 13.0 – 18.8% in the partial waxy genotypes (Table 5.1). Similar to the trend observed for amylose concentration, a higher protein concentration was recorded for single null genotypes (15.5 – 18.8%) than double null genotypes (13.0 – 14.7%). Wx-$D^-$ had significantly high ($p < 0.05$, 18.8%), while Wx-$A^-D^-$ had significantly low ($p < 0.05$, 13.0%) protein concentration.

### 5.4.4 Lipid concentration

Wx-$A^+B^+D^+$ genotype showed 2.3% lipid concentration, while it was highest (2.8%) in Wx-$A^-B^-D^-$ genotype (Table 5.1), agreeing with a previous report in barley (Asare *et al*., 2011). In addition, a negative correlation was observed between lipid and amylose (r = -0.61, $p < 0.01$) (Table 5.3), represented by higher lipid concentration in Wx-$A^-B^-D^-$ line.

### 5.4.5 Beta-glucan concentration

Beta-glucan concentrations in the partial and completely waxy genotypes varied from 0.6 – 0.9%, being 0.7% in Wx-$A^+B^+D^+$ genotype (Table 5.1). Different cereal grains were analyzed for their whole grain and endosperm β-glucan contents, and very low (0.23 – 0.36%) concentration for wheat endosperm was reported (Henry, 1987). In the present study, Wx-$A^-B^-D^-$ genotype recorded significantly ($p < 0.05$) higher concentration of β-glucan (1.1%) than the partial waxy genotypes. A similar pattern was observed in a previous study in barley, where a waxy cultivar, CDC Fibar, with low starch and amylose concentrations, had higher β-glucan concentration (Asare *et al*., 2011).

### 5.4.6 Amylopectin chain length distribution

A typical chain length distribution curve can be divided into seven regions: DP 6-8 (R-I), DP 9-11 (R-II), DP 12-14 (R-III), DP 15-18 (R-IV), DP 19-22 (R-V), DP 23-36 (R-VI) and DP 37-45 (R-VII) depending on its changing nodes (Figure 5.2). Differences were observed in R-I, which had comparatively more chains of DP 6-8 in wheat starch with complete GBSSI complement than the partial and completely waxy starches (Table 5.2). Similarly, subtractive

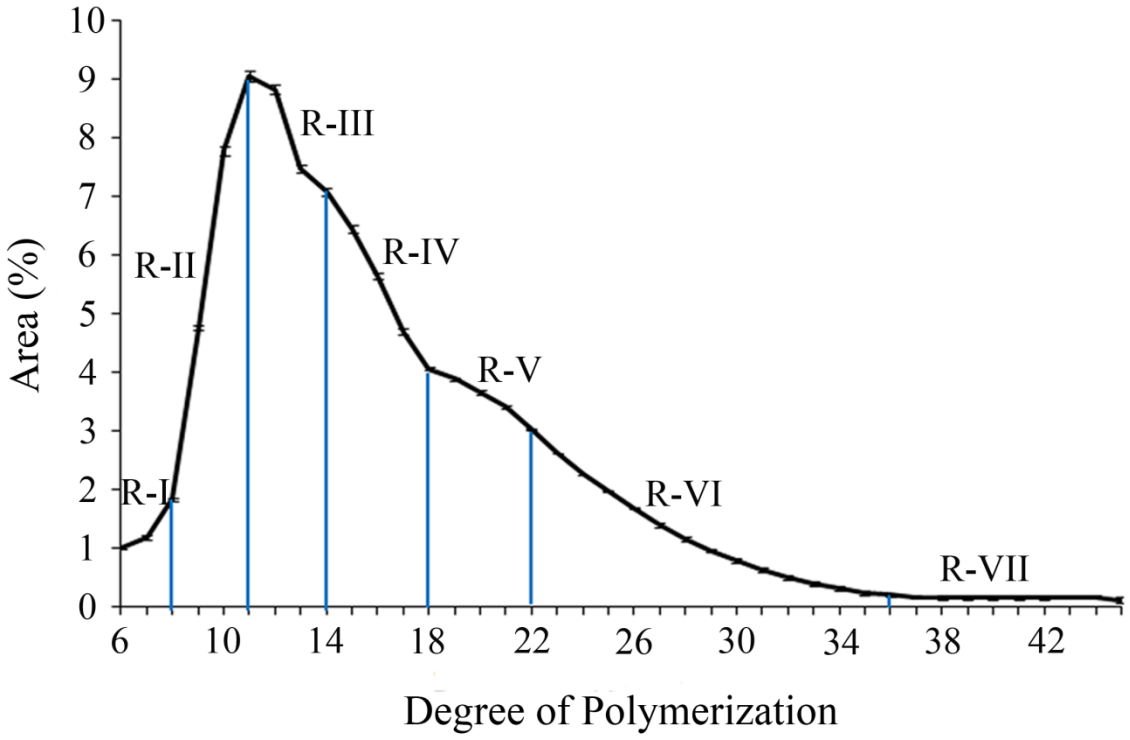



Figure 5.2 Amylopectin chain length distribution for starch extracted from non-waxy wheat genotype

The amylopectin unit chains were divided into seven regions: R-I (DP 6-8), R-II (DP 9-11), R-III (DP 12-14), R-IV (DP 15-18), R-V (DP 19-22), R-VI (DP 23-36), R-VII (DP 37-45), on the basis of changing slopes of the curve.

Table 5.2 Amylopectin chain length distributions[1] in wheat starch

| Genome composition | Distribution (%) | | | | | | | Average DP |
|---|---|---|---|---|---|---|---|---|
| | R – I (DP 6-8) | R- II (DP 9-11) | R – III (DP 12-14) | R – IV (DP 15-18) | R – V (DP 19-22) | R – VI (DP 23-36) | R – VII (DP 37-45) | |
| $A^+B^+D^+$ | 4.18 ±0.03$^{b}$ | 21.99 ±0.25$^{a}$ | 23.37 ±0.23$^{a}$ | 20.43 ±0.11$^{a}$ | 13.93 ±0.06$^{a}$ | 14.96 ±0.38$^{a}$ | 1.14 ±0.17$^{a}$ | 16.19 ±0.12$^{a}$ |
| $A^-B^+D^+$ | 3.78 ±0.04$^{ab}$ | 21.69 ±0.23$^{a}$ | 23.46 ±0.19$^{a}$ | 20.68 ±0.11$^{a}$ | 14.24 ±0.11$^{a}$ | 15.19 ±0.35$^{a}$ | 0.96 ±0.11$^{a}$ | 16.23 ±0.09$^{a}$ |
| $A^+B^-D^+$ | 3.79 ±0.04$^{ab}$ | 21.50 ±0.14$^{a}$ | 23.22 ±0.11$^{a}$ | 20.66 ±0.14$^{a}$ | 14.34 ±0.18$^{a}$ | 15.30 ±0.24$^{a}$ | 1.21 ±0.15$^{a}$ | 16.33 ±0.07$^{a}$ |
| $A^+B^+D^-$ | 3.58 ±0.13$^{a}$ | 21.98 ±0.20$^{a}$ | 23.65 ±0.32$^{a}$ | 20.68 ±0.10$^{a}$ | 14.36 ±0.09$^{a}$ | 14.81 ±0.39$^{a}$ | 0.93 ±0.08$^{a}$ | 16.17 ±0.08$^{a}$ |
| $A^-B^-D^+$ | 3.79 ±0.05$^{ab}$ | 21.15 ±0.16$^{a}$ | 23.07 ±0.14$^{a}$ | 20.54 ±0.09$^{a}$ | 14.22 ± 0.09$^{a}$ | 15.91 ±0.26$^{a}$ | 1.32 ±0.12$^{a}$ | 16.47 ±0.07$^{a}$ |
| $A^-B^+D^-$ | 3.82 ±0.06$^{ab}$ | 21.10 ±0.20$^{a}$ | 23.06 ±0.20$^{a}$ | 20.62 ±0.09$^{a}$ | 14.23 ±0.04$^{a}$ | 15.90 ±0.33$^{a}$ | 1.28 ±0.15$^{a}$ | 16.45 ±0.10$^{a}$ |
| $A^+B^-D^-$ | 3.86 ±0.14$^{ab}$ | 21.48 ±0.11$^{a}$ | 23.29 ±0.20$^{a}$ | 20.60 ±0.08$^{a}$ | 14.01 ±0.08$^{a}$ | 15.47 ±0.21$^{a}$ | 1.29 ±0.08$^{a}$ | 16.37 ±0.04$^{a}$ |
| $A^-B^-D^-$ | 3.73 ±0.10$^{a}$ | 21.02 ±0.38$^{a}$ | 22.90 ±0.30$^{a}$ | 20.48 ±0.30$^{a}$ | 14.33 ±0.10$^{a}$ | 16.20 ±0.70$^{a}$ | 1.35 ±0.39$^{a}$ | 16.53 ±0.24$^{a}$ |

[1] Values are based on average of three replications ± standard error. Mean values within a column followed by different superscripts are significantly different ($p < 0.05$), with superscript 'a' representing the smallest value.

graphs between Wx-$A^+B^+D^+$ and other waxy-null starches showed that non-waxy wheat had higher content of short chains of DP 6-10 compared to GBSSI-deficient starches (Figure 5.3). Chains of DP 6-10 occupied ~15% more area in the wheat starches with a complete complement of GBSSI proteins (Wx-$A^+B^+D^+$) compared to all other partial and completely waxy starches (Figure 5.3). Among the waxy null starches studied, Wx-$D^-$ showed ~10% more area for DP 6-10 than Wx-$A^-$ and Wx-$B^-$ (Figure 5.3). For other regions of chain length distribution, Tukey's test however revealed no clear differences among various waxy-null combination genotypes (Table 5.2). No significant differences were observed for average degree of polymerization as well.

### 5.4.7 Starch granule size distribution

Starch granule size showed bimodal distribution in Wx-$A^+B^+D^+$ and the waxy-null starches. Starch granule diameter ranged from 1.5 – 52.5 μm, with maximum volume occupied by granules of 17.4 μm diameter (Figure 5.4 A, B). On the basis of diameter, starch granules can be divided into C-granules (< 5 μm), B-granules (5 – 15 μm) and A-granules (> 15 μm) (Raeker *et al*., 1998). Wx-$A^-B^-D^-$ starch showed the highest volume percentage of C-granules and lowest volume percentage of A-granules among non-waxy and waxy-null starches (Figure 5.4 C). Wx-$A^-B^-$ and Wx-$D^-$ starches showed similar results i.e. reduced and increased volume percentage of C-granules and A-granules, respectively. This suggests a comparable role of Wx-D individually and Wx-A, Wx-B together in influencing the starch granule size distribution. Presence of Wx-D, either individually or in combination with Wx-A and/or Wx-B showed an increase in the volume percentage of A-granules (Figure 5.4 C). However, absence of Wx-D along with Wx-A or Wx-B increased the B- and C-granule volume (%) and reduced A-granule volume (%) (Figure 5.4 C). Normal distribution of number percent of starch granules can be divided into three portions; (i) d(0.1) – 10% of granules smaller than diameter set-point, (ii) d(0.5) – 50% of granules smaller than diameter set-point, and (iii) d(0.9) – 90% of granules smaller than diameter set-point. Values for d(0.1) in Wx-$A^-B^-D^-$ starch were significantly higher than those for Wx-$A^+B^+D^+$ (data not shown), suggesting the influence of waxy allele on starch granule size distribution.

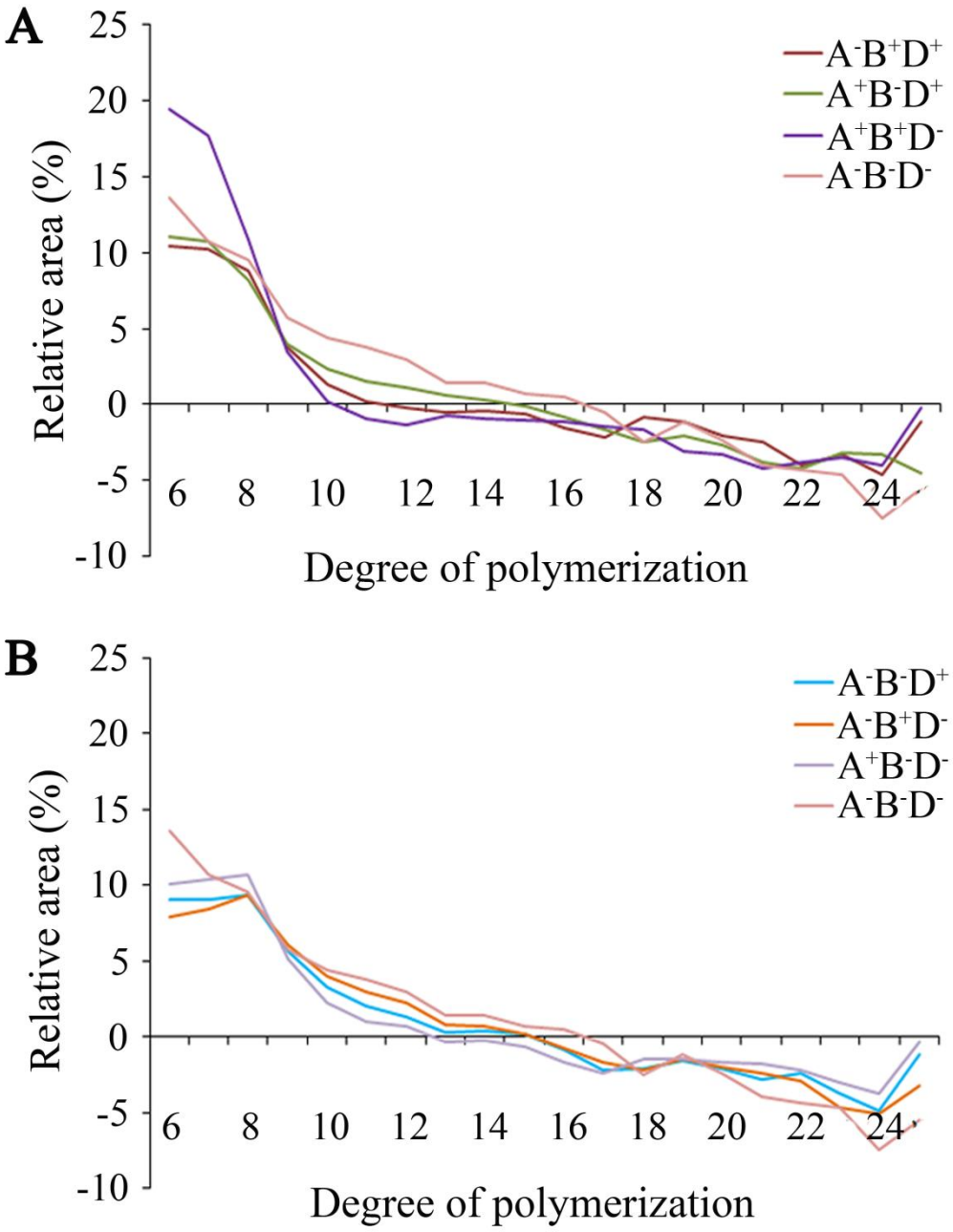
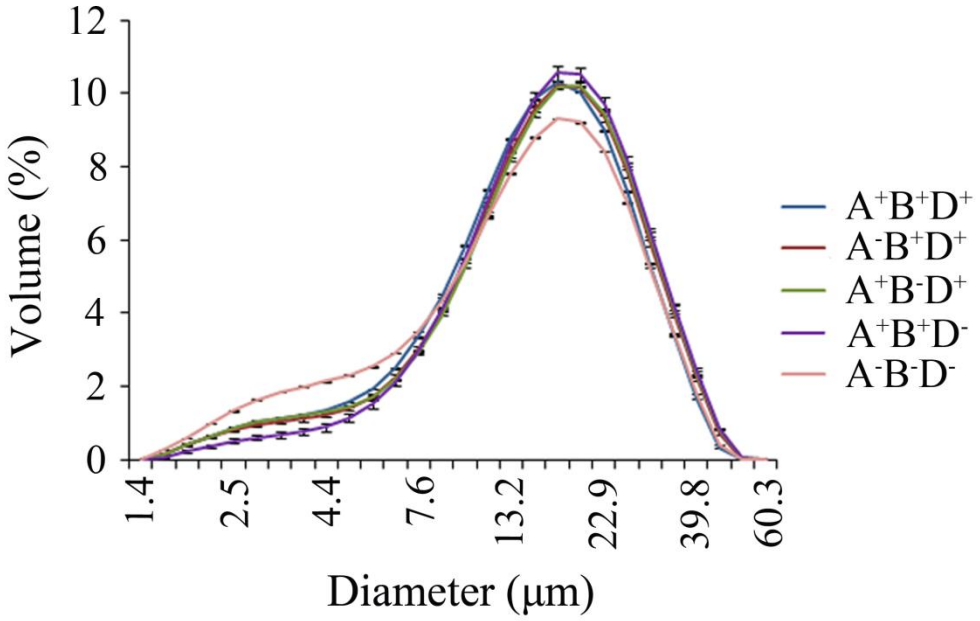


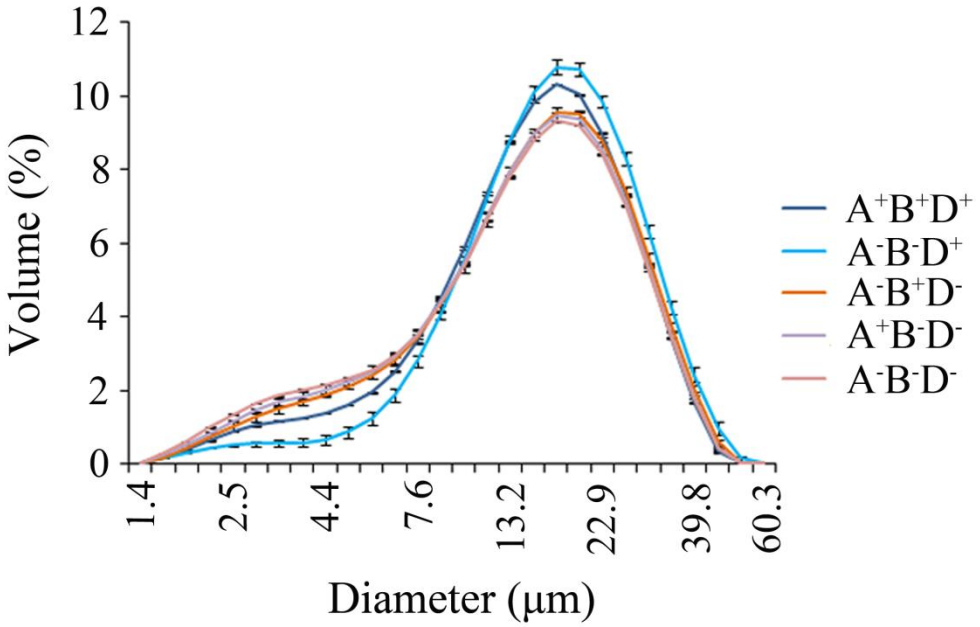

Figure 5.3 Amylopectin chain length distribution profile in partial and completely waxy wheat starch and non-waxy wheat starch

(A) Single waxy-null genotypes. (B) Double waxy-null genotypes. Percent relative chains were obtained by subtracting the area percentage occupied by chains of waxy-null starch from that of Wx-$A^+B^+D^+$ starch. Values are based on average of three replications.

A


12
10
8
6
4
2
0
Volume (%)
1.4
2.5
4.4
7.6
13.2
22.9
39.8
60.3
Diameter (μm)
A+B+D+
A-B+D+
A+B-D+
A+B+D-
A-B-D-

B


12
10
8
6
4
2
0
Volume (%)
1.4
2.5
4.4
7.6
13.2
22.9
39.8
60.3
Diameter (μm)
A+B+D+
A-B-D+
A-B+D-
A+B-D-
A-B-D-

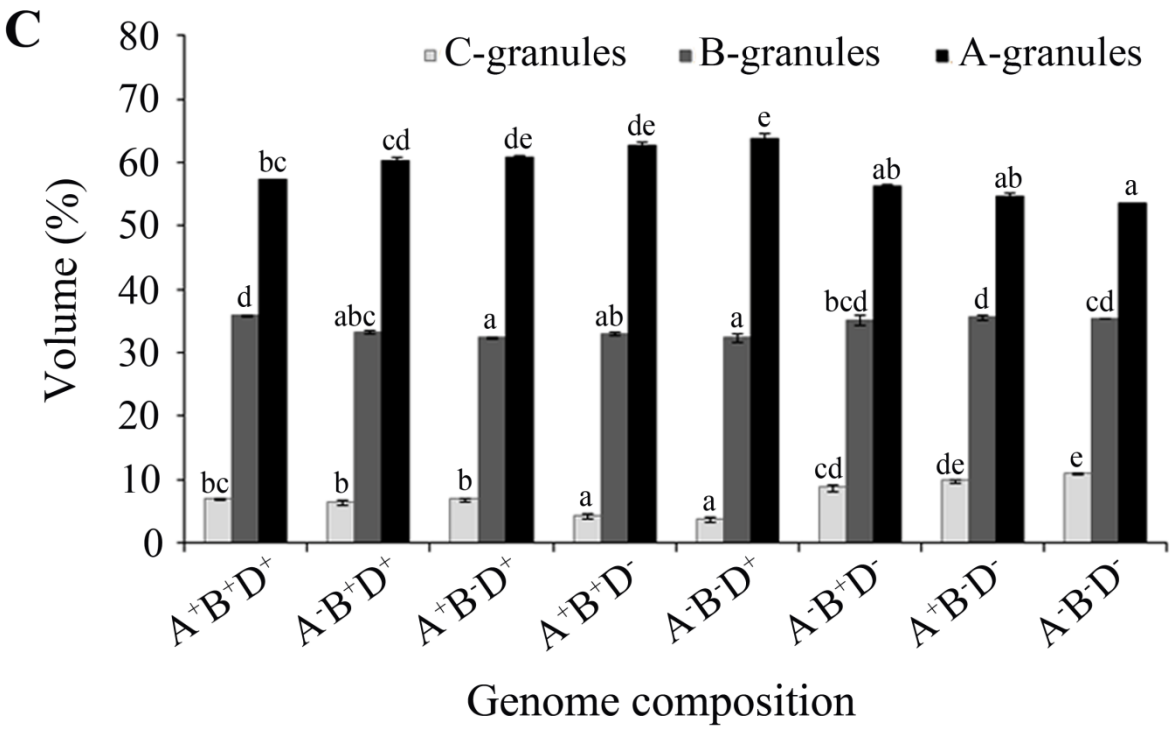



Figure 5.4 Starch granule size distribution in wheat endosperm starch

Line graphs showing the volume occupied by starch granules of different diameters in (A) single waxy-null genotypes and (B) double waxy-null genotypes. (C) Effect of the absence of genome specific GBSSI on C-type (< 5 μm), B-type (5 – 15 μm) and A-type (> 15 μm) diameter range of starch granules. Different letters on same colored columns indicate they are significantly different ($p < 0.05$) with 'a' representing the smallest value. Values represent means of three replications ± standard error.

### 5.4.8 *In vitro* starch enzymatic hydrolysis

The kinetics of hydrolytic studies in meal and pure starch showed a rapid increase in starch hydrolysis till 30 min, which is considered as Readily Digestible Starch (RDS). From 30-120 min, there was a slow increase in the rate of starch hydrolysis, which is considered as Slowly Digestible Starch (SDS). After 120 min, starch hydrolysis in pure starch reached saturation and was completely digested by 240 min. This portion of starch is known as Resistant Starch (RS) (Englyst *et al.*, 1992). In meal samples, however, the rate of starch hydrolysis was slower than in purified starch (Figure 5.5).

Percent soluble starch in white bread reached ~90% within 30 min as compared to non-waxy and partial waxy genotypes, which reached ~25% (meal) and 40-45% (pure starch) in the same time period (Figure 5.5). Completely waxy genotype showed a significantly higher rate of starch hydrolysis than all partial waxy genotypes, reaching ~40% and ~60% in meal and pure starch samples respectively within 30 min of incubation with hydrolytic enzymes. For meal samples of partial waxy genotypes, percent soluble starch increased linearly throughout the experiment duration, however reaching saturation at 120 min in the completely waxy genotype. For pure starch samples, the rate of hydrolysis was significantly higher than meal. In the completely waxy, pure starch samples reached saturation in 90 min compared to 120 min in meal samples (Figure 5.5).

In wheat grain meal, endogenous components like protein, lipid, and β-glucan are present in addition to starch. Completely waxy genotypes showed the highest rate of hydrolysis, reaching 50% hydrolysis in ~45 – 50 min, while other genotypes reached a similar stage after ~120 min. RDS in completely waxy genotype was significantly ($p < 0.05$) higher (24.8%) compared with non-waxy (12.9%) and partial waxy genotypes (11.0 – 16.0%). RS in completely waxy genotype was significantly ($p < 0.05$) lower (3.1%) than non-waxy (13.4%) and partial waxy genotypes (11.7 - 27.6%). SDS values in different genotypes ranged from 61.4 to 74.5%, with no specific pattern. Wx-$B^-D^-$ genotypes showed the lowest concentration of RDS and highest concentration of RS (Figure 5.6).

To investigate the influence of GBSSI on starch hydrolysis, purified starch was used to remove the confounding effects of other endogenous grain constituents. Hydrolysis curves

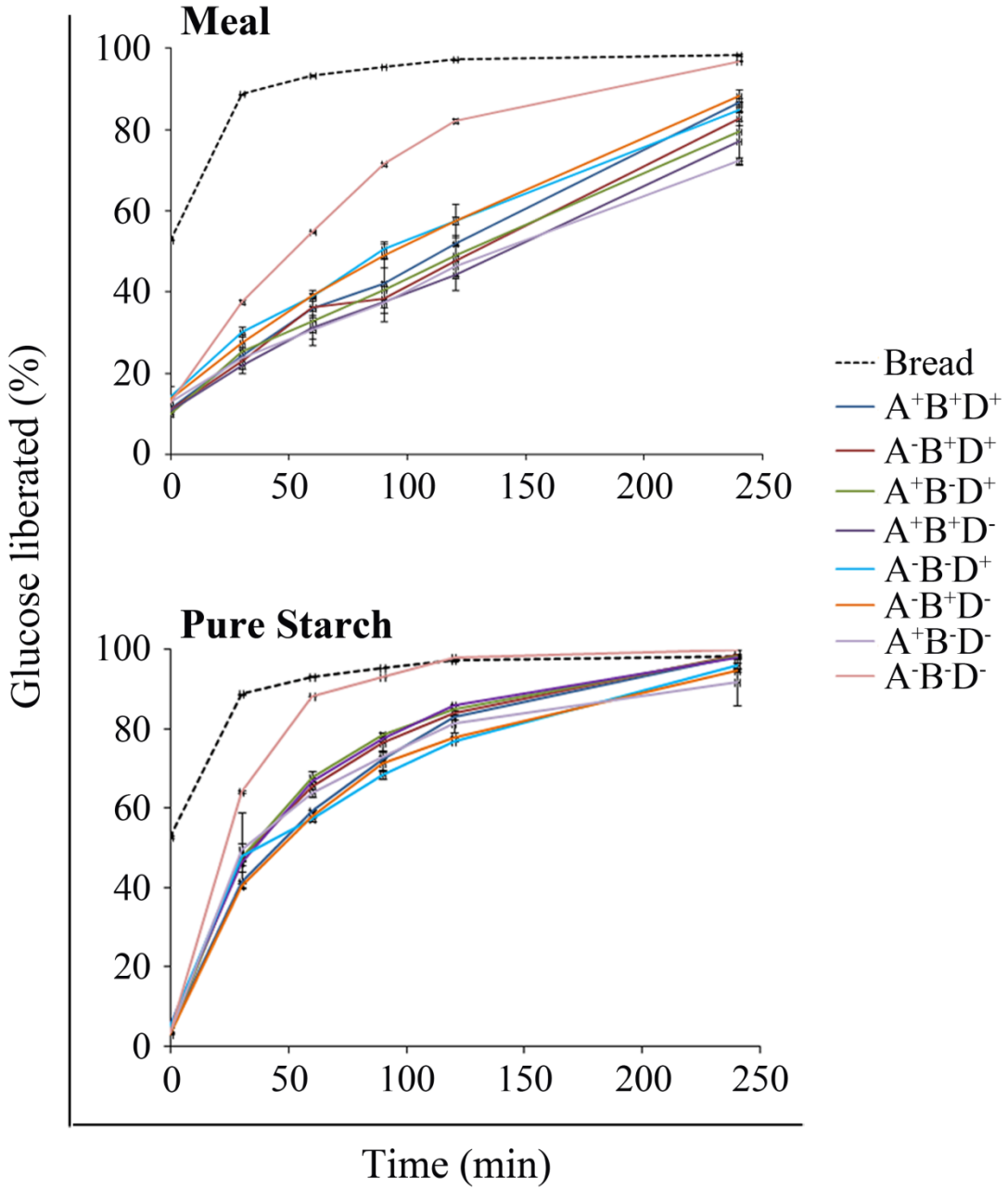



Figure 5.5 Rate of starch enzymatic hydrolysis curve in meal and extracted starch at selected time intervals in non-waxy, partial and completely waxy genotypes

Values were calculated on dry weight basis and represent the means of three biological replications and three independent observations for each replicate ± standard error.

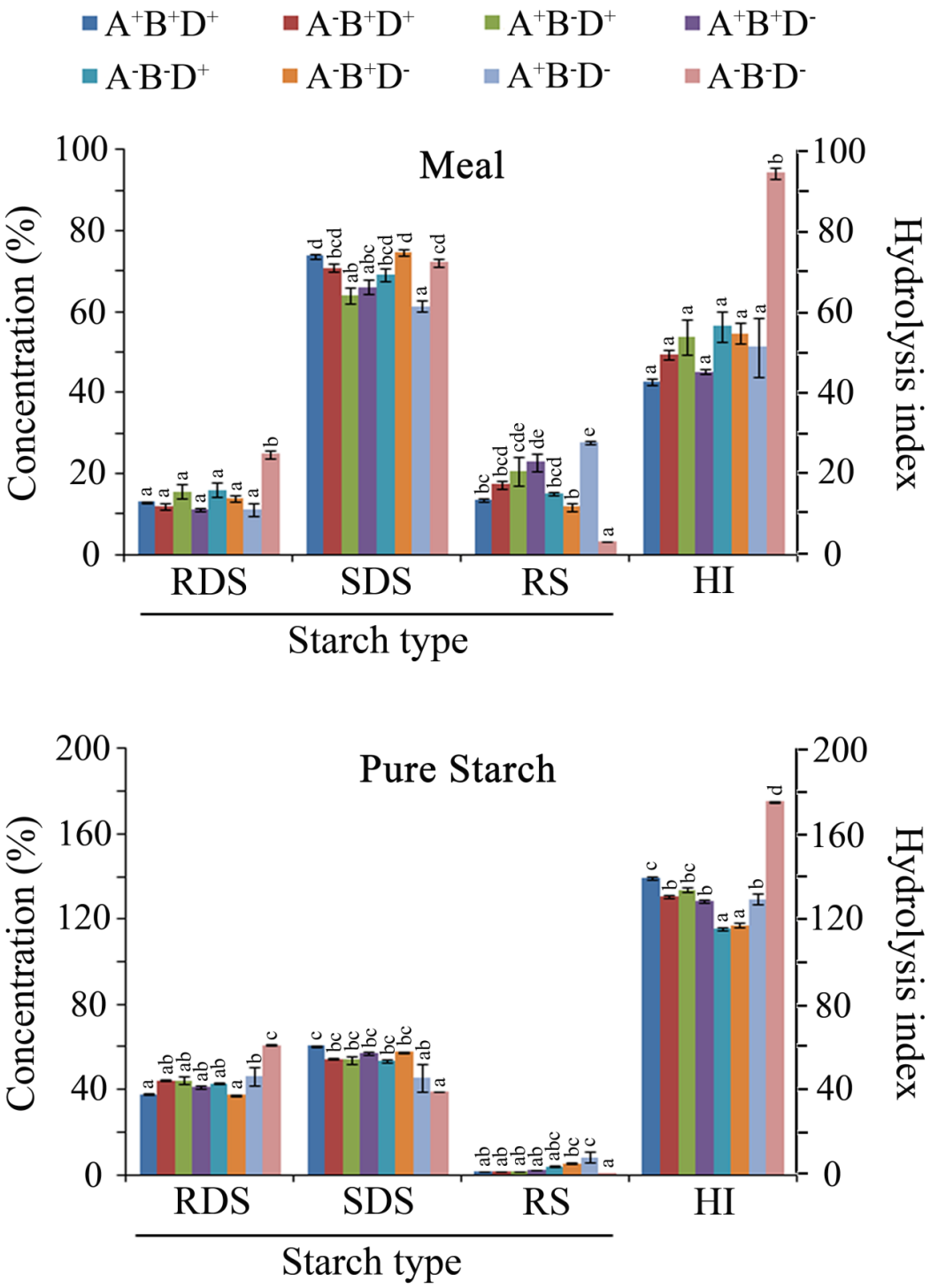



Figure 5.6 Enzymatic starch hydrolysis parameters of meal and pure starch from non-waxy, partial waxy and completely waxy genotypes

RDS, SDS and RS are percent values. HI is an absolute value. Values were calculated on dry weight basis and represent the means of three biological replications and three independent observations for each replicate ± standard error.

indicated that 50% of pure starch was hydrolyzed between 30-60 min in all genotypes. Similar to the meal, completely waxy genotypes had the highest amounts of RDS (60.9%), followed by non-waxy and partial waxy genotypes. RS concentration was lowest in the completely waxy genotypes (0.2%) (Figure 5.6). Consistent with the pattern of hydrolysis in meal, the highest RS concentration was also found in Wx-B$^{-}$D$^{-}$ genotypes with pure starch hydrolysis assay.

Hydrolysis index (HI) for meal was highest in the completely waxy genotype (94.3). The non-waxy genotype had a HI of 42.7. HI (pure starch) was also highest in the completely waxy genotype (175.0), while it ranged from 117.1 to 139.3 in non-waxy and partial waxy genotypes (Figure 5.6).

## 5.5 Discussion

### 5.5.1 GBSSI in relation with grain components accumulation

Wheat flour composition influences its properties and end-use. Amylose concentration and amylopectin structure are an integral part of starch functionality. In bread baking, substitution of non-waxy wheat flour with ~10% waxy wheat flour results in a higher loaf volume, while more than 30% substitution can lead to loss of granule rigidity and fusion of starch granules (Purna *et al*., 2011). In the present study, GBSSI double null genotypes had significantly higher total starch concentrations (62.9 – 64.8%) than single null genotypes (57.7 – 60.5%) (Table 5.1), without any significant effect on TGW. In a recent study in sorghum, *GBSSI* gene-associated single nucleotide polymorphic (SNP) markers affected starch content, independent of amylose concentration (Hill *et al*., 2012). In addition to starch, a dose dependent effect of Wx protein on amylose concentration was observed (Table 5.1), concurring with a previous report (Demeke *et al*., 1999). The gradual reduction in amylose concentration from non-waxy to two *Wx* gene null genotypes can be explained by the ability of the remaining functional gene to partially compensate for the missing *Wx* allele, as suggested previously (Geera *et al*., 2006). However, over-expression of *Wx* genes does not affect amylose content, despite the increase in the amount of Wx protein (Itoh *et al*., 2003; Sestili *et al*., 2012). This suggests an evolutionary limitation of the amylose threshold, above which it becomes inert to *Wx* gene dosage. However, considering previous reports and the present results it can be suggested that in wheat, genome-specific GBSSI activity is compensatory for amylose synthesis.

On the basis of catalytic potential for amylose synthesis, Wx-A1a was lowest, whereas Wx-B1a and Wx-D1a had similar activity (Miura *et al*., 2002). Wx-A1 and Wx-B1 proteins have been reported to be additive in action, while Wx-D1 is independent and strongest among all the GBSSI isoproteins in wheat endosperm (Debiton *et al*., 2010). Various reports have suggested a non-additive behaviour of Wx proteins, with interaction action being the dominant character influencing amylose concentration (Wickramsinghe and Miura, 2003). Although the order of contribution towards amylose synthesis is reported to be Wx-B1 ≥ Wx-D1 > Wx- A1 (Miura *et al*., 2002) and/or Wx-D1 > Wx-B1 > Wx-A1 (Debiton *et al*., 2010), no such pattern was observed in the present study (Table 5.1). Variants of *Wx* alleles have been studied in the past for their amylose synthesis ability with the following postulated order: Wx-A1b (null) = Wx-A1e < Wx-A1c < Wx-A1a < Wx-B1c = Wx-B1d ≤ Wx-D1a < Wx-D1c ≤ Wx-B1e (Yamamori, 2009). These variations in amylose synthesizing capacity of genome-specific Wx protein support the idea of regulatory mechanisms between genomes. However, this trait has also been reported to be influenced by growing environment and genetic background (Panozzo and Eagles, 1998), which could be the reason for differing results reported in the literature.

Lipid concentration was highest in the completely waxy genotype as compared to non-waxy and partial waxy genotypes (Table 5.1). The presence of amylose-lipid inclusion complexes has been reported in wheat (Yasui *et al*., 1996). Crystallinity studies (Yoo and Jane, 2002) with waxy wheat did not report the formation of such complexes, due to the absence of amylose (Graybosch, 1998). Non-waxy wheat varieties have lipid integrated with amylose. In the present study a lower concentration of free lipids was observed in non-waxy and partial waxy genotypes.

### 5.5.2 GBSSI in relation with amylopectin fine structure

The role of Wx protein in starch synthesis has been extensively studied in different plant systems. Previous studies have suggested an indispensible role of functional *Wx* allele either for synthesis of intermediates in amylose production or for the synthesis of long chain fraction of amylopectin (Ral *et al*., 2006). On the other hand, a study with wheat showed that starch synthase and starch branching enzymes are not regulated by the *Wx* null alleles (Debiton *et al*., 2010), suggesting that amylopectin formation pathway is not affected by lack of one, two or three *Wx* alleles. It has been shown that amylopectin structure is identical in waxy and their

respective parents in wheat near-isogenic lines (Yasui *et al*., 1996; Miura *et al*., 2002). However, in the present study, minor but significant ($p < 0.05$) differences were observed in the very short chain fraction, with Wx-$A^+B^+D^+$ showing higher concentration of DP 6-8 chains than Wx-$A^-B^-D^-$ (Table 5.2). Among the partial waxy starches, Wx-$A^+B^+D^-$ showed relatively higher concentration of short chains of DP 6-8 (Figure 5.3). However, Wx-A or Wx-B individually did not show such phenotype, thus suggesting an additive contribution of Wx-A + Wx-B towards the short-chain phenotype of waxy amylopectin. Although absence of Wx-D singly (Wx-$A^+B^+D^-$) showed a relative increase in DP 6-8 chains content, but the presence of Wx-D with Wx-A or Wx-B did not influence the short chain fraction (Figure 5.3), thereby suggesting a suppressive role of Wx-D towards the formation of short chains of waxy starch amylopectin. Significant ($p < 0.05$) differences were not observed in medium or long chain regions between non-waxy and partial or completely waxy genotypes (Table 5.2). Hence the results suggest that reduced GBSSI dosage subtly affects short glucan chain synthesis in amylopectin, however it is not a limiting factor for the development of amylopectin structure.

### 5.5.3 GBSSI in relation with starch granule size distribution

Granule size distribution of wheat starch is influenced by genotype (Stoddard, 2003) and environment (Liu *et al*., 2011). Granule size is an important characteristic that influences composition of starch and its functionality. Studies on starch granule sizes from various plant sources suggest their correlation with physico-chemical properties such as thermal, visco-elastic properties, gelatinization behaviours, pasting properties, swelling power, and water-binding capacity, both in non-waxy and waxy genotypes (Graybosch, 1998; Lindeboom *et al*., 2004).

In the present study, starch granule size showed bimodal distribution in non-waxy, partial waxy and completely waxy starch (Figure 5.4 A, B). However in previous studies, waxy maize starch showed altered starch granule size distribution compared to non-waxy maize starch (Franco *et al*., 1998; Cardoso and Westfahl Jr., 2010). The completely waxy starch in this study had the highest volume percentage of small C-type starch granules and lowest volume percentage of large A-type starch granules, compared to non-waxy and partial waxy starch (Figure 5.4 C). Similarly, in previous reports, the volume percentage of small starch granules was shown to be higher in waxy starch compared to the non-waxy starch in wheat (Bertolini *et al*., 2003) and barley (Asare *et al*., 2011). Additionally, a decrease in large A-type starch granule

content with increase in waxy character has been reported (Geera *et al*., 2006). It has been suggested to be a secondary effect of Wx protein dosage, which could occur by either alteration of glucan chains thereby reducing starch debranching enzyme activity, or a pleiotropic effect of *Wx* gene on other starch biosynthetic enzymes, hence altered large and small starch granule properties (Geera *et al*., 2006). In the present study, significant ($p < 0.05$) differences in starch granule size distribution between non-waxy and completely waxy starch were observed, concurring with a previous study on waxy wheat (Geera *et al*., 2006). Contrastingly, in few other studies, differences in starch granule size distribution between waxy and non-waxy wheat varieties have not been observed (Yoo and Jane, 2002).

### 5.5.4 GBSSI in relation with *in vitro* starch enzymatic hydrolysis

Grain and starch characteristics affect the starch digestibility of cereal grains (Asare *et al*., 2011). Amylose is a negative regulator of starch digestibility (Regina *et al*., 2006). The reason for the characteristic of amylose lies in its linear hydrogen-bond stabilized double-helical structure, which is resistant to enzymatic hydrolysis. Recently, production of barley amylose-only starch granules resulted in 65% RS for gelatinized starch, which was 2.2-fold higher than for non-waxy starch (Carciofi *et al*., 2012). In the present study, Pearson's bivariate correlation analysis showed a negative correlation of amylose with hydrolysis index (r = -0.77, $p < 0.01$) and RDS (r = -0.61, $p < 0.01$); and a positive correlation with RS (r = 0.49, $p < 0.01$) (Table 5.3). This is also reflected in the completely waxy genotype, having low amylose concentration and high hydrolysis index, as compared to non-waxy and partial waxy starch.

Correlation between starch characteristics showed that amylose was positively correlated with large A-type starch granules (r = 0.49, $p < 0.01$) and negatively correlated with small C-type starch granules (r = -0.54, $p < 0.01$) (Table 5.4), which is in agreement with a previous report (Raeker *et al*., 1998), but differed from a recent report in barley (Asare *et al*., 2011). In addition, large A-type starch granules were negatively correlated with hydrolysis index (r = -0.46, $p < 0.01$) and small C-type starch granules were positively correlated with hydrolysis index (r = 0.50, $p < 0.01$) and negatively correlated with SDS (r = -0.36, $p < 0.05$) (Table 5.4). Larger size, lenticular shape, higher amylose (Peng *et al*., 1999) and long chains of amylopectin (Ao and Jane, 2007) in A-type starch granules could be the reasons for their reduced digestibility. The

Table 5.3 Correlation analysis between grain constituents and starch enzymatic hydrolysis parameters

| | TGW | Starch | Amylose | Protein | Lipid | β-Glucan | RDS M | SDS M | RS M | HI M |
|---|---|---|---|---|---|---|---|---|---|---|
| TGW | 1 | -0.25 | 0.48* | 0.65** | -0.28 | -0.65** | -0.36 | -0.41* | 0.49* | -0.37 |
| Starch | | 1 | -0.05 | -0.74** | -0.13 | 0.35** | -0.24 | 0.18 | 0.01 | -0.19 |
| Amylose | | | 1 | 0.31* | -0.61** | -0.85** | -0.61** | -0.18 | 0.49** | -0.77** |
| Protein | | | | 1 | -0.01 | -0.63** | -0.13 | -0.32* | 0.32* | -0.22 |
| Lipid | | | | | 1 | 0.46** | 0.30* | -0.15 | -0.06 | 0.41** |
| β-Glucan | | | | | | 1 | 0.58** | 0.28 | -0.55** | 0.69** |
| RDS M | | | | | | | 1 | 0.12 | -0.68** | 0.92** |
| SDS M | | | | | | | | 1 | -0.81** | 0.05 |
| RS M | | | | | | | | | 1 | -0.59** |
| HI M | | | | | | | | | | 1 |

*. Significance at $p < 0.05$. **. Significance at $p < 0.01$.

Table 5.4 Correlation analysis between starch characteristics and starch enzymatic hydrolysis parameters

| | Starch | Amylose | C-granules | B-granules | A-granules | RDS PS | SDS PS | RS PS | HI PS | R - I | R - II | R - III | R - IV | R - V | R - VI | R - VII | Avg. DP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Starch | 1 | -0.05 | 0.01 | 0.05 | -0.03 | -0.25 | 0.07 | 0.33* | -0.40** | 0.25 | -0.20 | -0.14 | -0.23 | -0.37* | 0.20 | 0.35* | 0.24 |
| Amylose | | 1 | -0.54** | -0.33* | 0.49** | -0.63** | 0.53** | 0.00 | -0.61** | 0.06 | 0.35* | 0.26 | 0.15 | 0.01 | -0.33* | -0.27 | -0.33* |
| C-granules | | | 1 | 0.70** | -0.95** | 0.35* | -0.36* | 0.17 | 0.50** | 0.16 | -0.30 | -0.30 | -0.03 | 0.07 | 0.23 | 0.23 | 0.24 |
| B-granules | | | | 1 | -0.89** | 0.11 | -0.18 | 0.22 | 0.32 | 0.24 | -0.17 | -0.20 | -0.15 | -0.15 | 0.17 | 0.23 | 0.19 |
| A-granules | | | | | 1 | -0.27 | 0.31 | -0.21 | -0.46** | -0.21 | 0.27 | 0.28 | 0.09 | 0.02 | -0.23 | -0.25 | -0.24 |
| RDS PS | | | | | | 1 | -0.92** | 0.19 | 0.76** | -0.35* | -0.12 | -0.08 | -0.16 | 0.05 | 0.21 | 0.09 | 0.18 |
| SDS PS | | | | | | | 1 | -0.57** | -0.53** | 0.40** | 0.15 | 0.08 | 0.17 | 0.04 | -0.26 | -0.11 | -0.22 |
| RS PS | | | | | | | | 1 | -0.26 | -0.27 | -0.10 | -0.03 | -0.08 | -0.20 | 0.20 | 0.09 | 0.17 |
| HI PS | | | | | | | | | 1 | -0.04 | 0.03 | -0.03 | -0.09 | 0.08 | 0.01 | 0.00 | 0.00 |
| R - I | | | | | | | | | | 1 | 0.09 | -0.15 | -0.05 | -0.24 | -0.17 | 0.09 | -0.13 |
| R - II | | | | | | | | | | | 1 | 0.91** | 0.38** | -0.42** | -0.89** | -0.75** | -0.93** |
| R - III | | | | | | | | | | | | 1 | 0.49** | -0.33* | -0.87** | -0.79** | -0.90** |
| R - IV | | | | | | | | | | | | | 1 | 0.34* | -0.68** | -0.65** | -0.65** |
| R - V | | | | | | | | | | | | | | 1 | 0.11 | -0.06 | 0.15 |
| R - VI | | | | | | | | | | | | | | | 1 | 0.75** | 0.96** |
| R - VII | | | | | | | | | | | | | | | | 1 | 0.89** |
| Avg. DP | | | | | | | | | | | | | | | | | 1 |

*. Significance at $p < 0.05$. **. Significance at $p < 0.01$.

reason for higher rate of hydrolysis in C-type starch granules could be attributed to more surface area or more free ends of glucan chains for amylases to act on.

To determine characteristics associated with starch specific and meal parameters, cluster dendrograms with average linkage correlation were constructed (Figure 5.7). For wheat grain meal, amylose and RS are parts of the same clade, suggesting a close association between the two components. For pure starch, RDS and small granules were present on branches of the same clade, suggesting a positive correlation between these variables. SDS was present close to amylose and large A-type starch granules. RS was located not too far from longer chains of amylopectin DP 23-45. This further supports that A-type starch granules, amylose, and longer chains of amylopectin play a substantial role in starch hydrolysis potential.

Genome specific GBSSI showed variations in starch characteristics and relation with starch hydrolysis. Absence of Wx-D showed an increase in short chains of amylopectin (Figure 5.3), whereas the presence of Wx-D either singly or in combination, showed reduced short chains of DP 6-8. This suggests a suppressive role of Wx-D in the formation of short chain fraction of amylopectin. In addition, presence of Wx-D, either singly or in combination, led to an increased volume percentage of large A-type starch granules (Figure 5.4 C). Moreover, individual Wx-D showed significantly ($p < 0.05$) lower hydrolysis index, while the rate of hydrolysis was higher when Wx-D was present with Wx-A or Wx-B (Figure 5.6). Therefore, it can be suggested that among the Wx isoproteins, Wx-D might be the major contributor influencing starch hydrolysis in wheat.

## 5.6 Conclusions

In conclusion, this study indicates dosage dependent amylose synthesis, where absence of one Wx protein reduces the amylose concentration by 1.3% and further absence of two Wx proteins reduces it to 22.4% from 28.7% in non-waxy wheat starch. It is further suggested that GBSSI is not limiting for amylopectin synthesis, because only subtle differences in amylopectin chain length distribution were observed in non-waxy, partial and completely waxy starches. Complete absence of Wx protein affects starch granule size distribution, with a 37% increase in C-type starch granules in completely waxy than non-waxy starch. The present study suggests that the absence of GBSSI affects starch granule growth by inhibiting glucan chain elongation. Rate of starch hydrolysis is significantly influenced by granule size, amylose concentration, and long

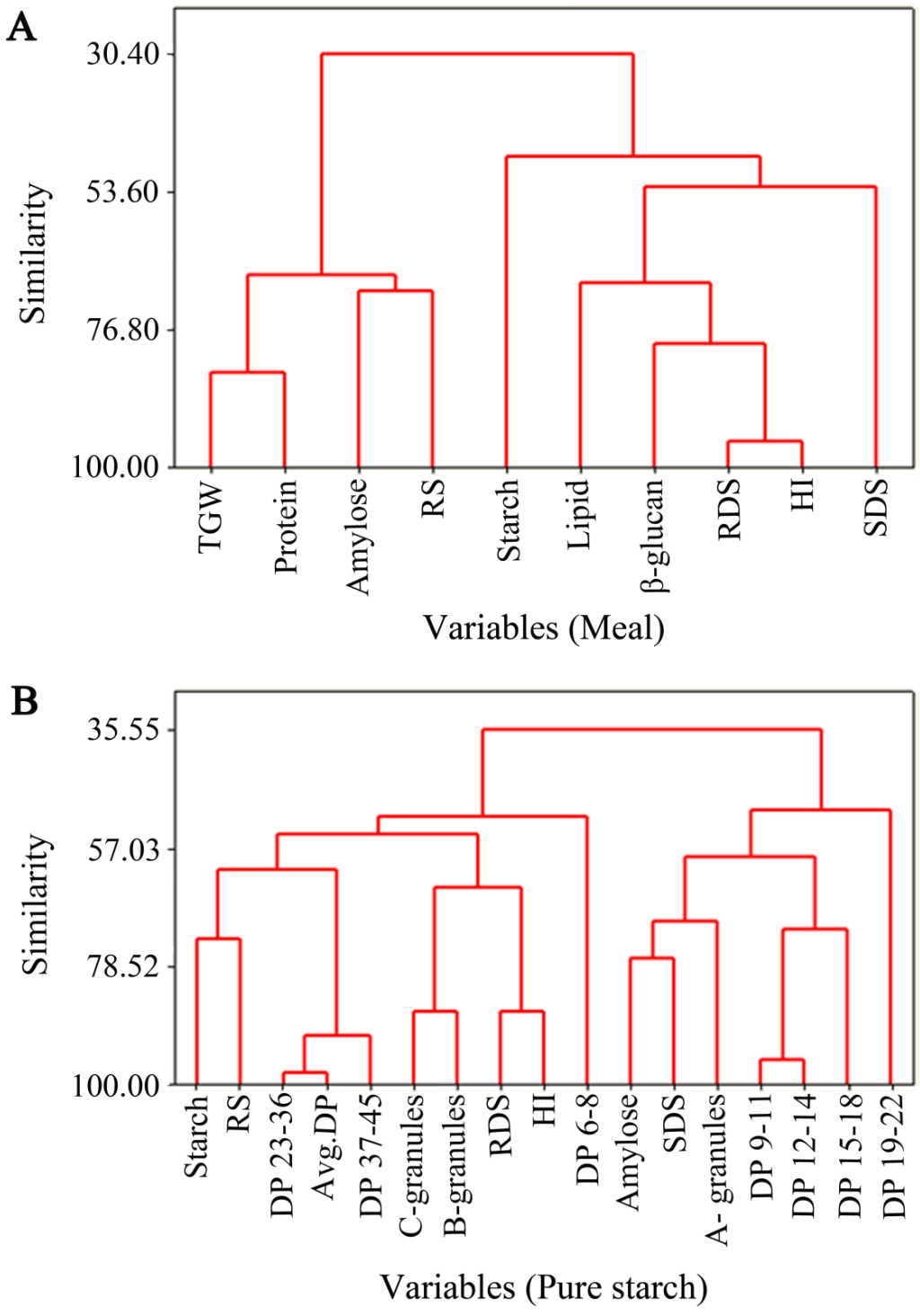



Figure 5.7 Average linkage dendrogram depicting physical relationship between different components of wheat grain related to starch enzymatic hydrolysis

(A) Meal characteristics (B) Starch characteristics

chain fraction of amylopectin (Figure 5.7). The above mentioned starch properties could be utilized to develop speciality starches for food, feed and industrial applications.

# 6. DIFFERENTIAL INFLUENCE OF WAXY PROTEIN ON STORAGE STARCH CHARACTERISTICS DURING WHEAT GRAIN DEVELOPMENT

**Study 3**[3]

Formerly in Study 2, starch characteristics were studied in mature grains of near-isogenic waxy-null wheat lines. Study 3 was conducted to analyze the effects of genome specific GBSSI on starch morphology, composition and structure during development. It was found out that the basic architecture of amylopectin is established by 12 DPA, and that it was influenced by different GBSSI isoproteins at different stages of development. Thus, this study provided an insight into the change in the various starch granules exterior and interior characteristics during development, which lead to the mature granule characteristics finally influencing *in vitro* starch hydrolysis as observed in Study 2.

[3] **Ahuja, G.**, Jaiswal, S., Hucl, P., & Chibbar, R.N. (2013). Differential influence of waxy protein on storage starch characteristics during wheat grain development. (To be submitted)

## 6.1 Abstract

Wheat grain development is a complex process and is characterized by changes in physicochemical and structural properties of starch. The present study deals with endosperm starch physicochemical properties and structure during development in different waxy-null genotypes. The study was conducted with pure starch isolated from wheat grains at 3-30 days post anthesis (DPA), at three day intervals. Polyacrylamide gel electrophoresis and immunoblotting of non-waxy and completely waxy genotypes showed the presence of GBSSI at 3 and 6 DPA in the waxy genotype. Amylose concentration increased throughout grain development in non-waxy and partial waxy genotypes. Completely waxy genotype showed ~7% amylose at 3 and 6 DPA, which declined during development and reached non-detectable amounts by 30 DPA. Amylopectin structure had a higher content of short chains at 3 DPA, which decreased continuously until 12 DPA, after which there were only minor changes in amylopectin chain length distribution. The average degree of polymerization was variable at different grain development stages, suggesting a non-consistent amylopectin synthesis during grain development. Wx-B and Wx-D affected amylopectin short chains mostly of DP 6-8 at 3 and 6 DPA. Wx-A affected the same fraction of chains at 9 and 12 DPA, and Wx-D affected DP 18-25 chains from 18 to 30 DPA; suggesting differential effect of waxy isoproteins on amylopectin structure formation.

## 6.2 Introduction

Starch is a glucan homo-polymer composed of one-quarter amylose (linear with few branches) and three-quarters amylopectin (highly branched). Linear chains are formed by α-1,4 glucosidic linkages, while branches are formed by α-1,6 glucosidic linkages. Structure of amylose and amylopectin, and their organization in the intact granule has been widely discussed (Buléon *et al*., 1998). Amylopectin shows a polymodal distribution of chains, organized into clusters, giving the molecule its architectural specificity (Hizukuri, 1985; 1986). Briefly, A-chains (DP 6 – 12) are the outermost chains, B-chains named B1, B2 and so on depending on their degree of polymerization (DP 13 – 24 up to 50) support A-chains or other B-chains. They are attached via α-1,6 glucosidic linkage, to the central C-chain, which within the granule is oriented towards the centre or hilum of the granule (Manners, 1989). Packing of these chains, or rather amylopectin molecule in the granule determines the basic structure of the granule.

The first step in the synthesis of amylose and amylopectin is brought about by ADP-glucose pyrophosphorylase (AGPase). Elongation and branching of amylopectin is a complex process and involves an array of enzymes viz., starch synthases (SS), starch branching enzymes (SBE) and debranching enzymes (DBE) (Myers *et al*., 2000). Elongation of amylose, however, is brought about solely by the enzyme; Granule-bound starch synthase I (GBSSI) or 'Waxy' protein. Plants lacking GBSSI are termed 'waxy'. In addition, GBSSI has been postulated to have a role in amylopectin synthesis as well (Ral *et al*., 2006). Amylopectin mutants of cereals and *Chlamydomonas* that lack GBSSI differ from that of non-waxy genotypes (Delrue *et al*., 1992). Incorporation of [$^{14}$C] glucose from ADP [$^{14}$C] glucose by GBSSI in granules from different species has been reported to be mainly into the long chain fraction of amylopectin (Denyer *et al*., 1996a). However, the precise contribution of GBSSI to amylopectin synthesis is still unclear.

Waxy wheat starch differs from non-waxy wheat in amylopectin structure. No obvious difference in the glucan chain-length distribution profiles of amylopectin molecules between waxy and non-waxy starch within three chain-length groups were observed (Yasui *et al*., 1996). In contrast different amylopectin chain length distributions have also been reported (Li Chun-Yan *et al*., 2007). These studies were conducted on mature grains. To better understand changes in amylopectin chain length distribution profiles, some studies have been conducted at different developmental stages. It was reported that the content of A- and short B-glucan chains increased in the waxy, and decreased in the non-waxy type of wheat during grain development. Also, the content of mid-length B-glucan chains decreased in both waxy and non-waxy starch during grain development. It was also suggested that GBSSI elongates the A- or short B-glucan chains to produce long B-glucan chains of amylopectin (Cao *et al*., 2012a).

Since wheat is an allohexaploid (2n = 6x = 42), it has three doses of GBSSI, one from each genome, namely Wx-A1, Wx-B1 and Wx-D1. Considering GBSSI potentially contributes to amylopectin synthesis or elongation, its absence from each genome could affect the glucan chain length distribution of amylopectin and/or organization of glucan chains within the granule, resulting in changes in the morphology of starch granules during wheat grain development. Therefore, the present study focused on studying starch characteristics during wheat grain development in non-waxy and waxy-null genotypes.

## 6.3 Materials and Methods

### 6.3.1 Plant material

Near-isogenic waxy null lines including four lines of each Wx-$A^+B^+D^+$, $A^-B^+D^+$, $A^+B^-D^+$, $A^+B^+D^-$, $A^-B^-D^+$, $A^-B^+D^-$, $A^+B^-D^-$ and $A^-B^-D^-$ were used for the developmental study. Plants were grown in a growth chamber in pots containing Redi-earth (W. R. Grace & Co. of Canada, ON, Canada) at 23 °C / 16 h light (350 μmol $m^{-2}$ $s^{-2}$ PPFD) and 19 °C / 8 h dark. Plants were fertilized every 3 weeks with a slow release fertilizer, Nutricote-14-14-14:N-P-K (Plant Products Co. Ltd., Brampton ON, Canada). Spikes, in triplicate, were tagged at anthesis, collected and frozen in liquid $N_2$ at selected days post anthesis (DPA), viz., 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 days, and stored at -80 °C for starch extraction. Spikes were collected in mid-afternoon consistently for all DPAs. Starch was purified from freeze-dried seeds from the middle of the spikes using a modified method (Peng *et al*., 1999) involving cesium chloride density gradient centrifugation (Asare *et al*., 2011).

### 6.3.2 Starch granule morphology by scanning electron microscopy

Prior to imaging under scanning electron microscope, starch granules were gold coated to make them electrically conductive. Starch granules were spread on, or attached to a metal stub with double-sided carbon tape and gold coated using sputter gold coater (S150B, Edwards, Wilmington MA, USA). Granule morphology was observed using a scanning electron microscope (SU6600, Hitachi High-Technologies Europe GmbH, Krefeld, Germany) at 5 kV.

### 6.3.3 Total starch concentration determination

Total starch concentration was determined using an AACC approved method (AACC method 76-13.01), in which ground meal samples were hydrolyzed to dextrins and finally D-glucose using α-amylase and amyloglucosidase respectively. Total starch concentration was determined as free glucose by measuring the absorbance at 510 nm (Hucl and Chibbar, 1996) and calculated on a percent dry weight basis (McCleary *et al*., 1997).

### 6.3.4 Amylose concentration determination by HPSEC

High performance size exclusion chromatography (HPSEC) was used to determine the amylose concentration (Demeke *et al*., 1999). Starch samples were debranched using 4 units isoamylase (200 U/mL, Megazyme International Ltd., Wicklow, Ireland) freeze-dried, suspended in dimethyl sulfoxide (DMSO) and injected (40 µL) into the column (PL gel MiniMix 250 x 4.6 mm ID column, Polymer Laboratories Inc., Amherst MA, USA) (Asare *et al*., 2011). Data was collected and analyzed using Empower 1154 chromatography software (Waters Corporation, Milford MA, USA).

### 6.3.5 Protein detection and immunoblotting

Proteins were extracted from the starch granules by suspending 10 mg starch in 300 µL denaturing electrophoresis buffer (Demeke *et al*., 1997). GBSSI was separated by polyacrylamide (15%, 1 mm thick gel) denaturing gel electrophoresis (Protean II, Biorad, Hercules CA, USA) (Demeke *et al*., 1997). Separated polypeptides were visualized by silver staining (Gromova and Celis, 2006). The gel-electrophoresis separated polypeptides were transferred on a nitrocellulose membrane at 10 V for 4 h (Gao and Chibbar, 2000). The membrane with transferred polypeptides was processed and incubated with primary antibodies raised against wheat GBSSI. The antigen-antibody complex was then detected with goat anti-rabbit alkaline phosphate conjugate using BCIP/NBT immunoscreening colour development kit (Biorad, Hercules CA, USA).

### 6.3.6 Amylopectin chain length distribution by capillary electrophoresis

Chain length distribution of amylopectin molecules was determined by fluorophore assisted capillary electrophoresis (FACE) (O'Shea *et al*., 1998) using Proteome Lab PA800 (Beckman Coulter, Fulerton CA, USA) equipped with a 488 mm laser module. A modified debranching protocol (Asare *et al*., 2011) was used to obtain unit amylopectin chains, which were labeled using 8-aminopyrene 1, 2, 6-trisulfonate (APTS) in the presence of sodium cyanoborohydride/ tetrahydrofuran. The N-CHO (PVA) capillary with pre-burned window (50 µm ID, 50.2 cm total length) was used to separate debranched samples.

### 6.3.7 Starch components morphology by atomic force microscopy

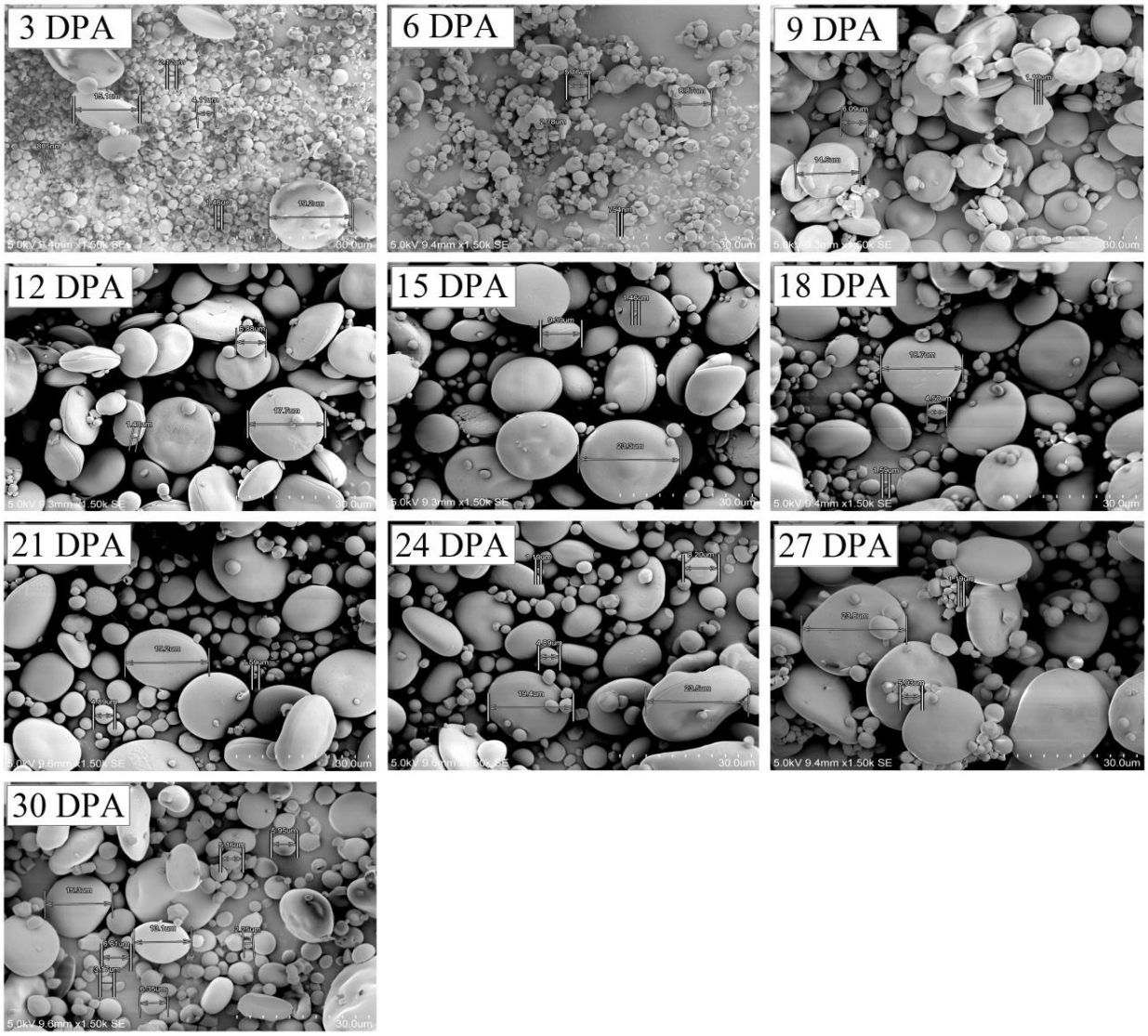
Starch solutions of concentration 30 µg/mL, kept at 90 °C, and were deposited onto freshly cleaved mica as an aerosol spray by nitrogen gas (Maley *et al*., 2010). AFM images were taken using a PicoSPM instrument (Molecular Imaging, Tempe, AZ) which operates in intermittent contact mode. The force constant on the silicon cantilever (Nanoscience Instrument, Phoenix AZ, USA), the resonant frequency and the curvature radius for AFM imaging were 48 $Nm^{-1}$, 190 kHz and < 10 nm respectively. The ratio of set-point oscillation amplitude to free air oscillation amplitude was 0.75:0.85 while resonance amplitudes ranged from 0.4 to 1.0 V (Maley *et al*., 2010). The instrument was under ambient conditions and mounted in a vibration isolation system with a scan rate 1-1.5 Hz (512 pixels per line). Analysis of images and measurements were conducted using SPIP V. 5.0.5 software (Image Metrology, Lyngby, Denmark).

## 6.4 Results

### 6.4.1 Starch granules morphology during grain development

Starch granule morphology was studied only in the non-waxy and completely waxy genotypes. In the non-waxy genotype, starch granules of all sizes ranging from 0.8 – 19.2 µm diameter were initiated at 3 DPA (Figure 6.1A). However in the waxy genotype, only small starch granules with diameter maxima of 5.0 µm were observed at this stage (Figure 6.1B). At 6 DPA, starch granule population was dominated by granules up to 11 µm (non-waxy) and 13 µm (waxy) diameters, in addition to smaller starch granules. At 9 DPA, equatorial grooves (as reported by Evers, 1971) were observed for large growing starch granules (Figure 6.1). In addition, small starch granules of size 1.2 µm (non-waxy, Figure 6.1A) and 0.8 µm (waxy, Figure 6.1B) were also observed. Between 12 and 15 DPA, starch granule diameter maxima reached 23.3 µm (non-waxy, Figure 6.1A) and 26.3 µm (waxy, Figure 6.1B), along with shallower equatorial grooves. Emergence of new population of small starch granules was observed between 18 and 21 DPA as depicted by increased starch granule density of diameter 1.6 µm (non-waxy, Figure 6.1A) and 1.7 µm (waxy, Figure 6.1B). After this stage, large starch granules did not increase in size and remained to a maximum of 22.5 µm for both non-waxy and waxy genotypes. Although both small and large starch granules were present at 30 DPA, the waxy genotype showed higher number of small starch granules than the non-waxy genotype.

A



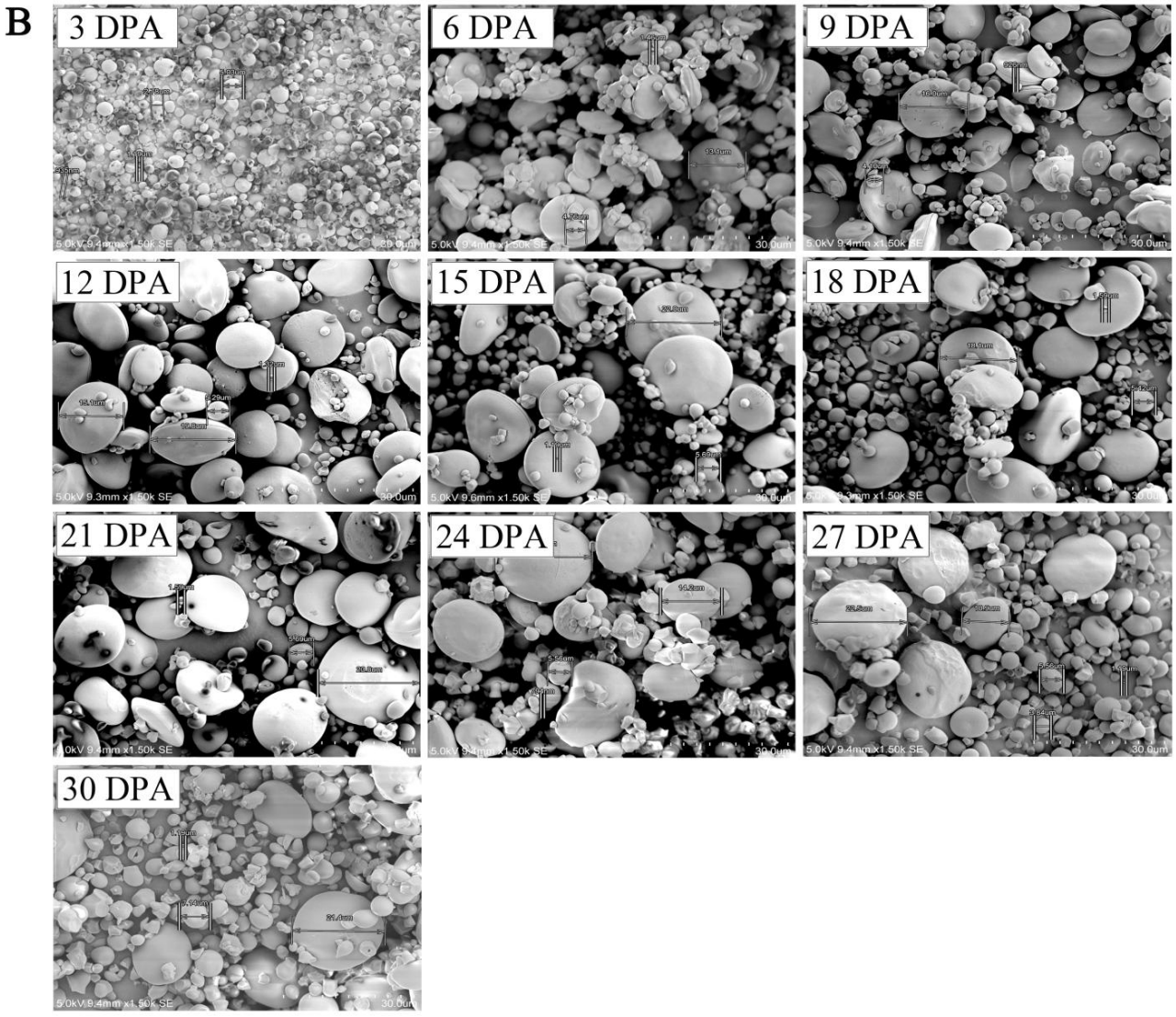



Figure 6.1 SEM images of starch granules isolated at different developmental stages

(A) Non-waxy (B) Completely waxy genotypes

### 6.4.2 Carbohydrate accumulation during grain development

Free glucose concentration in the non-waxy genotype was 3.2% at 3 DPA which decreased to 0.2% at maturity. Linear decrease in free glucose concentration, with minor differences, was recorded for all partial waxy and completely waxy genotypes (data not shown), concurring with observations in *Brachypodium* (Guillon *et al*., 2011).

Starch accumulation increased from 3 to 30 DPA i.e. from 8.3 to 66.2% and from 3.5 – 5.4 to 53.2 – 66.2% for non-waxy and partial waxy genotypes respectively. Total starch concentration in the completely waxy genotype increased until 21 DPA (3.9 – 48.0%), after which it reached saturation, being 50.3% at 30 DPA. Rate of total starch accumulation depicted by linear regression values, in waxy genotype ($r^2 = 0.90$) was higher than non-waxy genotype ($r^2 = 0.86$), although the non-waxy genotype showed a significantly higher starch concentration throughout development (Figure 6.2). From 3 to 6 DPA, starch concentration in Wx-A$^-$ genotypes doubled, while a small increase was observed in other genotypes. This suggests that Wx-A could be playing a regulatory role in starch accumulation. At 15 and 21 DPA, starch concentration was higher in single nulls than double nulls, suggesting a significant influence of GBSSI dosage on starch accumulation.

Amylose accumulation sigmoid-curve during granule development showed a linear increase from 3 to 6 DPA, remained constant till 9 DPA, increased exponentially from 12 to 18 DPA, and reached stationary phase after 21 DPA (Figure 6.3). In the non-waxy genotype, amylose concentration increased from 7.2% at 3 DPA to 30.5% at 30 DPA. The rate of amylose accumulation among the partial waxy genotypes differed during grain development. From 6 to 9 DPA, it was higher in the single nulls ($r^2 = 1.07$) than double nulls ($r^2 = 0.24$); from 9 to 18 DPA, it was similar for all the genotypes ($r^2 = 1.08 – 1.09$). Thereafter single nulls again had a higher concentration of amylose than double nulls, reaching 29.2% and 24.6% respectively at 30 DPA. Exception to this trend was the completely waxy genotype, which showed a consistent reduction in the rate of amylose accumulation throughout grain development (7.0 – 0.8%) (Figure 6.3).

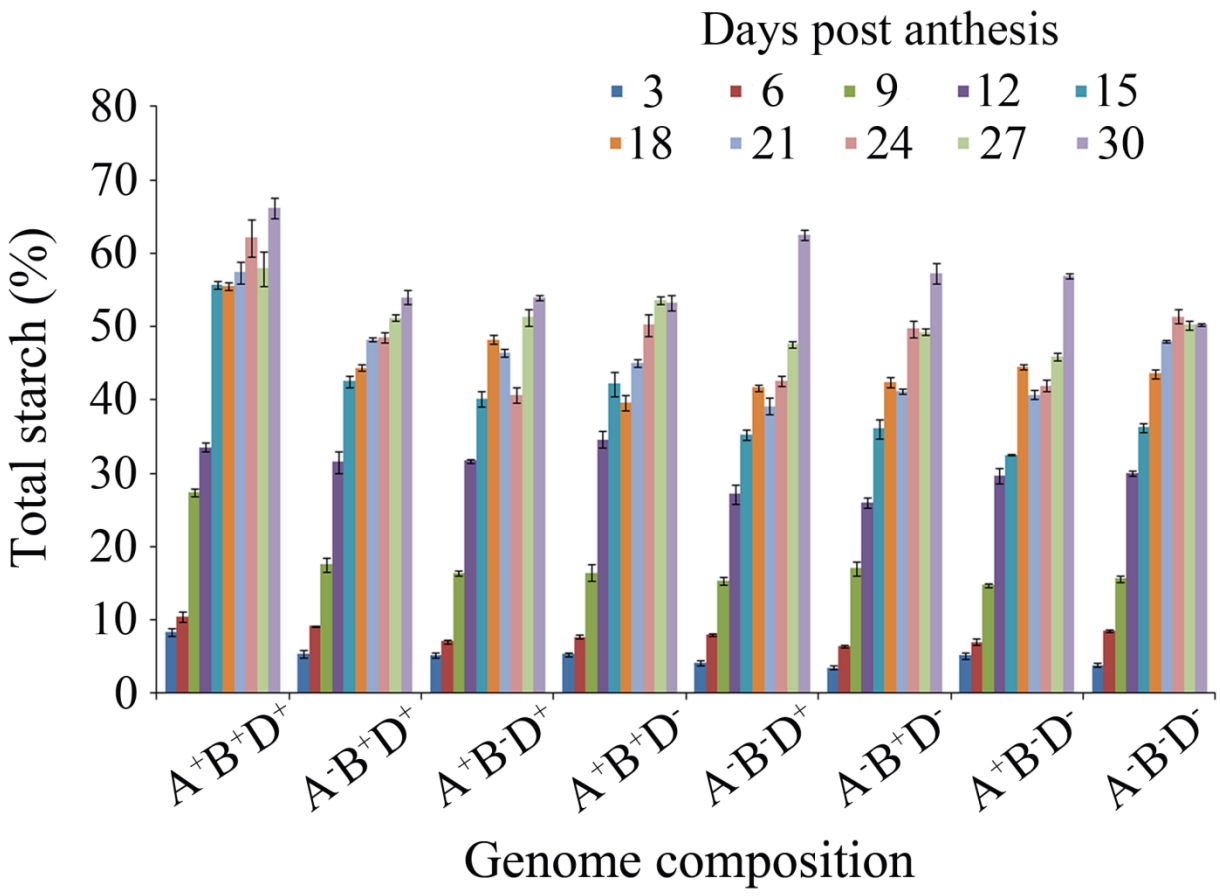



Figure 6.2 Total starch concentration {dry weight (%)} in non-waxy and waxy-null genotypes during wheat grain development

Values are means of three replications ± standard error.

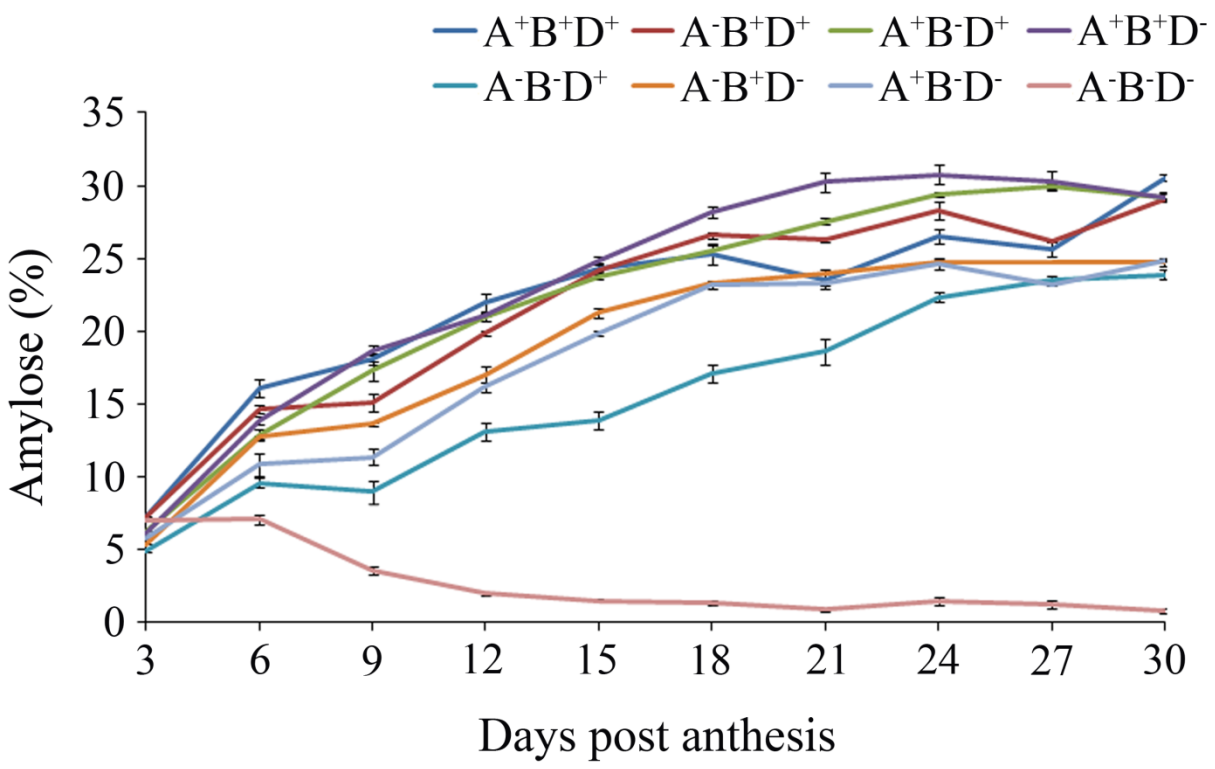



Figure 6.3 Amylose concentration in non-waxy and waxy-null genotypes during wheat grain development

Values are means of three replications ± standard error.

### 6.4.3 Starch granule polypeptides during grain development

Denaturing polyacrylamide gels were run for non-waxy and completely waxy genotypes to determine whether the amount of GBSSI accumulated within starch granules correlated with amylose content. For the non-waxy wheat genotype, reduced accumulation of GBSSI (~60 kDa) was observed at 3 and 6 DPA, while normal accumulation levels were observed from 9 to 30 DPA (Figure 6.4A). In the non-waxy genotype, the level of accumulation of SSII (100-115 kDa), SBE (87 kDa) and SSI (77 kDa) was lower until 9 DPA, after which a constant level of accumulation of these proteins was observed. Very little accumulation of SBEIc (152 kDa) was observed from 18 – 30 DPA. In the waxy genotype, a similar level of accumulation of SSII, SBE and SSI was observed throughout grain development. SBEIc was found to be completely missing in the waxy genotype. As expected for the waxy genotype, GBSSI accumulation was observed at 3 and 6 DPA, but not from 9 to 30 DPA (Figure 6.4B). Presence of GBSSI was confirmed by immunoblot analysis using polyclonal antibodies raised against wheat GBSSI (Figure 6.5). Since starch synthesizing enzymes in cereals have been shown to work in multi-protein complexes (Tetlow *et al*., 2008), immunoblotting was performed for other starch synthesizing enzymes to check whether the absence of GBSSI affects the accumulation of other starch granule proteins. SSI, SSII, SBEI and SBEII showed a similar pattern in non-waxy and waxy genotypes indicating that absence of GBSSI did not affect the accumulation of other starch granule-bound proteins (Figure 6.5).

### 6.4.4 Amylopectin chain length distribution during grain development

Significant changes were observed in the amylopectin chain length distribution pattern at different stages of grain development. Most variation was observed from 3 to 12 DPA, after which there were no considerable differences in amylopectin chain length distribution till 30 DPA (Figure 6.6). In all genotypes, glucan chain degree of polymerization (DP) < 14 decreased significantly from 3 to 9 DPA; while glucan chains of DP > 19 increased from 6 to 12 DPA. Glucan chains of DP 15 – 18 did not show considerable variation during grain development. In the non-waxy genotype, glucan chains of DP 6-8, DP 9-11 and DP 12-14 decreased from 6.2%, 30.7% and 38.9% at 3 DPA to 3.7%, 25.2% and 32.9% at 9 DPA respectively. Glucan chains of DP 15-18 did not show significant variation across development stages, being 24.2% at 3 DPA,

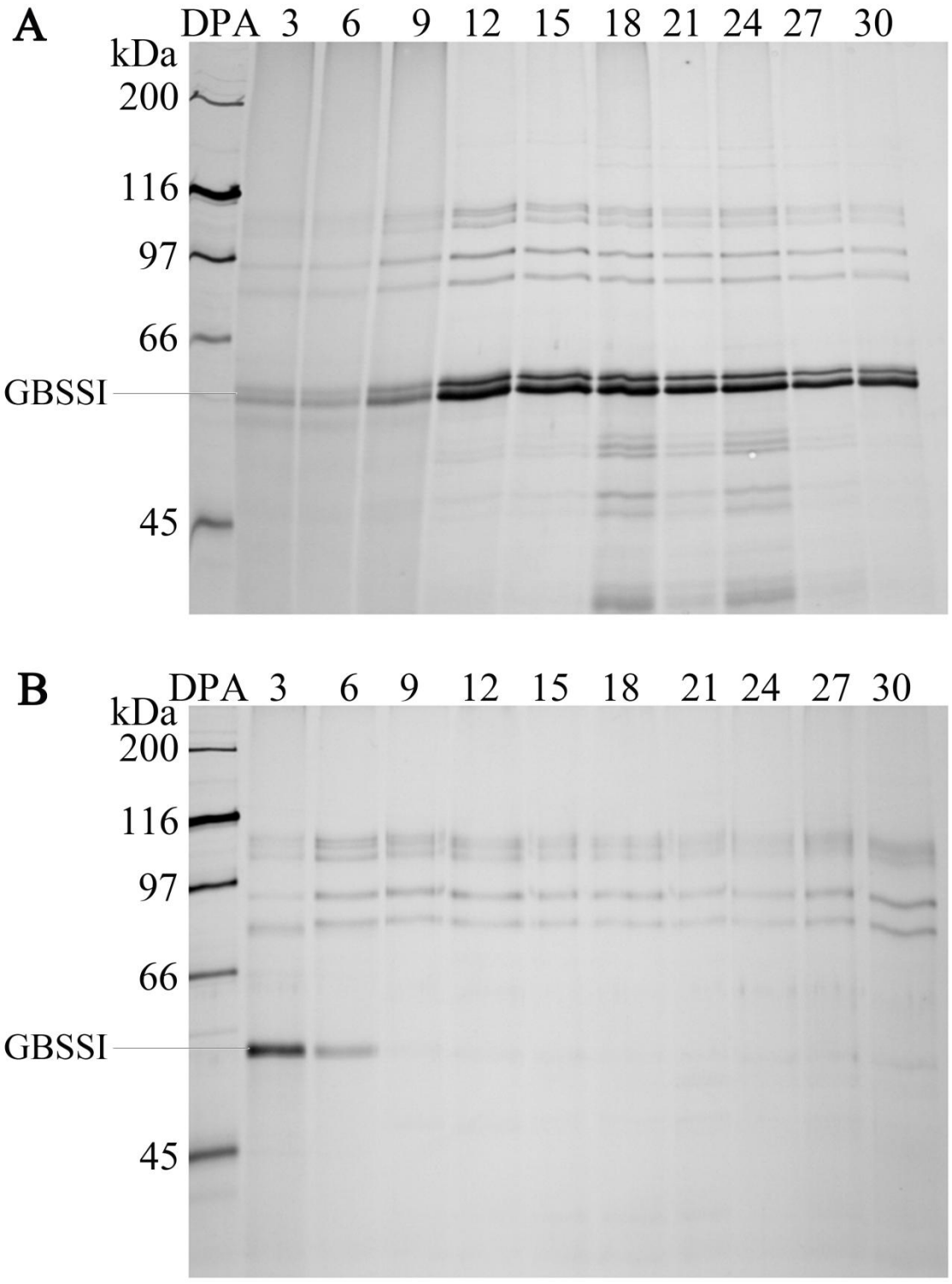



Figure 6.4 Denaturing gels showing the presence of starch granule-bound proteins at different development stages

(A) Non-waxy (B) Completely waxy genotypes

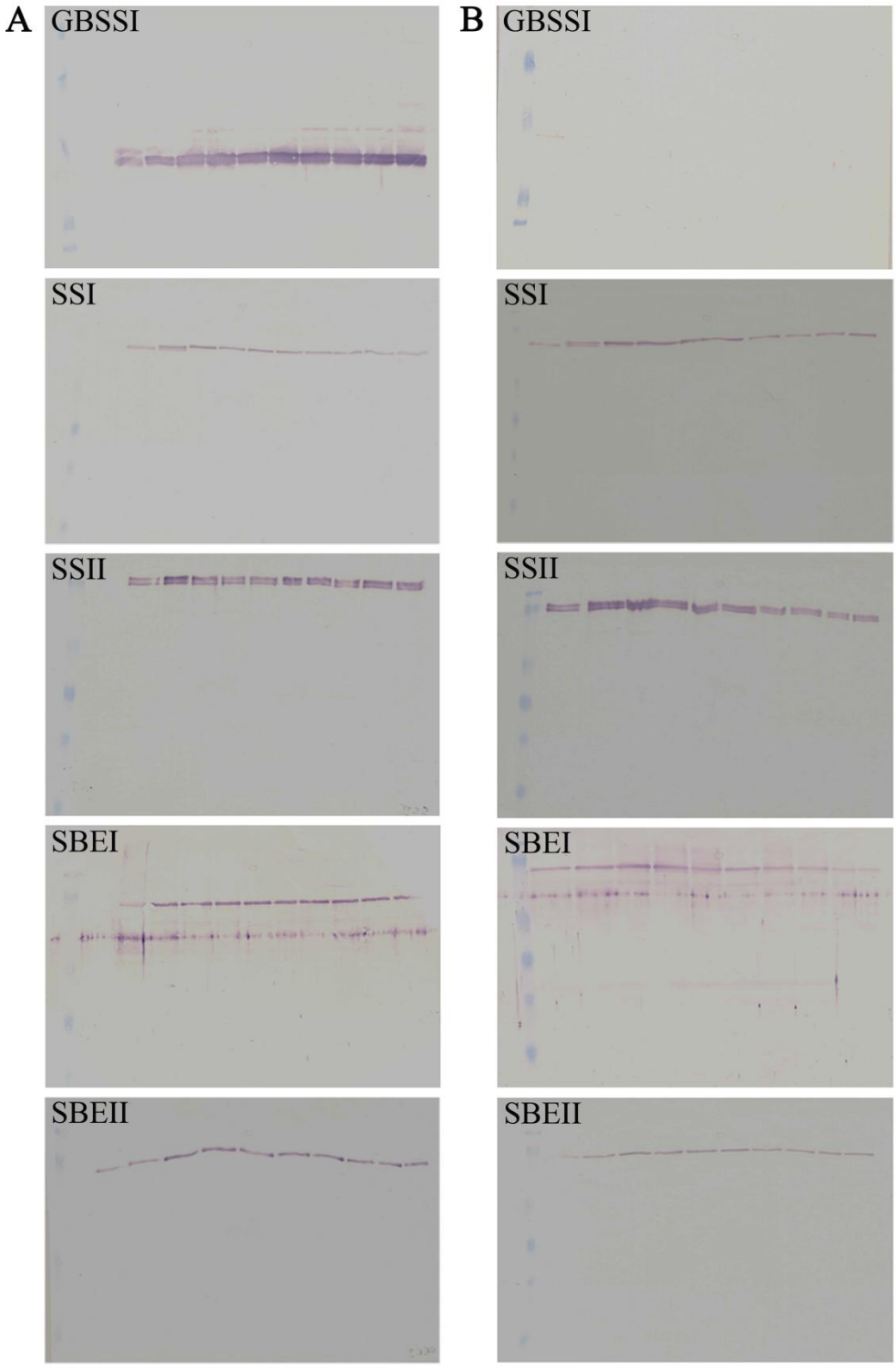



Figure 6.5 Immunoblots confirming the presence of GBSSI, SSI, SSII, SBEI and SBEII at different development stages

(A) Non-waxy (B) Completely waxy genotypes

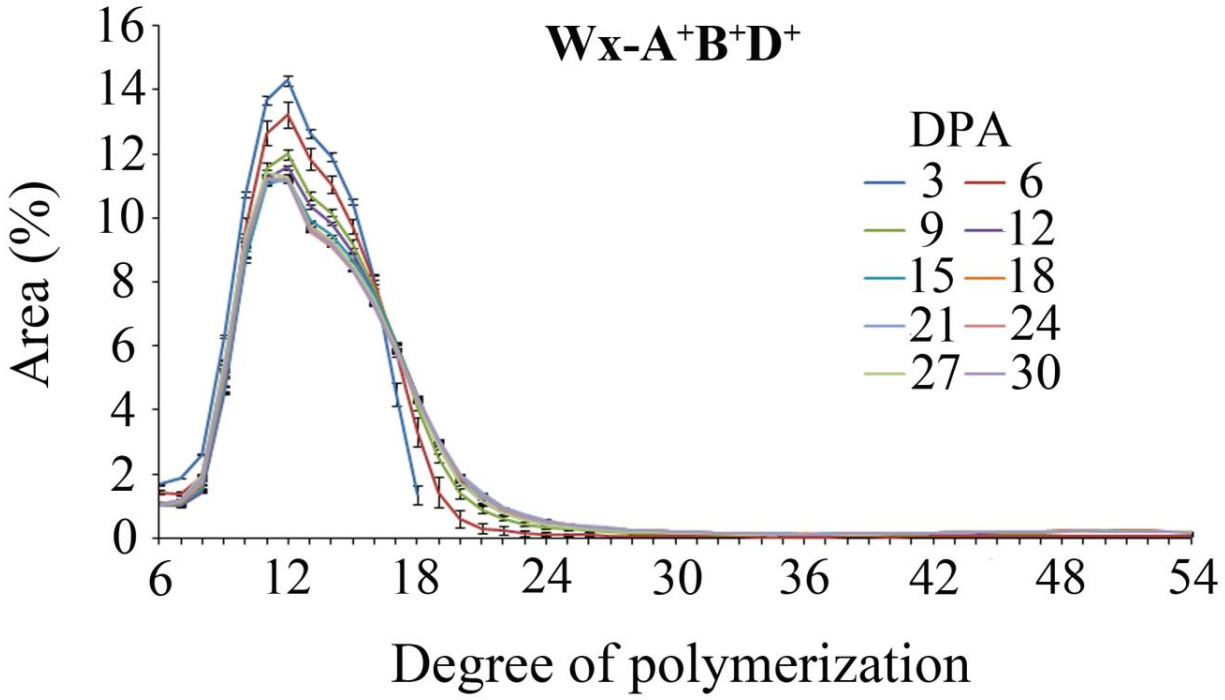



Figure 6.6 Amylopectin chain length distribution during wheat grain development in the non-waxy genotype

Values are means of three replications ± standard error.

27.1% at 12 DPA and 25.9% at 30 DPA. Glucan chains of DP 19-22, DP 23-36 and DP 37-45 increased from 2.6%, 0.9% and 1.1% at 6 DPA to 6.6%, 3.6% and 3.1% at 12 DPA respectively. Glucan chains of DP ≥ 19 were absent at 3 DPA (data not shown). These results were also reflected in the increase in average DP from 3 to 9 DPA and no change thereafter (Table 6.1). Changes in amylopectin glucan chain length distribution during development suggest that there is a continuous change in the length and arrangement of glucan chains up till at least 12 DPA (Figure 6.6), after which granules attain a specific shape, hence the average DP values do not differ significantly. Subtle differences were observed in the amylopectin glucan chain length distribution with the reduction of waxy dosage during wheat grain development (Figure 6.7). Variation was observed in the glucan short chain fraction (DP 6-8) of all the waxy-null starches. At 3 DPA, glucan short chains of DP 6-8 were affected most by Wx-B, Wx-D and Wx-BD followed by Wx-AB, Wx-AD and least affected by Wx-A (Figure 6.7). At 6 DPA, the largest effect on DP 6-8 glucan chains was observed for Wx-BD, which reduced the number of glucan chains longer than DP 9. At this stage, Wx-AB, Wx-B and Wx-D showed a similar effect, followed by Wx-A and Wx-AD. Wx-AD displayed a considerably higher proportion of DP 15-17 glucan chains than even the non-waxy starch. At 9 DPA, Wx-AB affected DP 6-15 glucan chains more as compared to other waxy-null starches, with Wx-A being the least effective. At 12 DPA, all partial waxy genotypes followed the same glucan chain length distribution pattern, except Wx-AB, which displayed a higher proportion of DP 6-8 glucan chains as compared to the non-waxy starch. At 18 DPA, the exception was Wx-D which showed more glucan chains of DP 6-8 than the non-waxy; while others followed the same pattern i.e. reduced glucan chains of DP 6-18 and increased proportion of glucan chains DP > 19. At 24 and 30 DPA, differences were observed in glucan chains of DP > 20 in Wx-A (Figure 6.7).

### 6.4.5 Starch components morphology

Atomic force microscopy for non-waxy and completely waxy genotypes showed differences in the height of fibrils (Table 6.2). In the non-waxy genotype at 3 DPA, no fibrils but nanoparticles were present with an average height of 1.6 nm (Figure 6.8A). At 12 and 30 DPA, fibrils had an average height of 0.6 nm each. Contour lengths of fibrils at 12 and 30 DPA were similar, being 180 ±129 nm and 180 ±164 nm respectively. However the poly-dispersity index

Table 6.1 Average degree of polymerization[1] of non-waxy, partial and completely waxy genotypes at different stages of grain development

| Genotype | Days post anthesis | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3 | 6 | 9 | 12 | 18 | 24 | 30 |
| $A^+B^+D^+$ | 12.3 ±0.2$^{a}$ | 13.5 ±0.3$^{b}$ | 14.7 ±0.1$^{c}$ | 15.0 ±0.1$^{c}$ | 15.1 ±0.1$^{c}$ | 15.0 ±0.0$^{c}$ | 15.0 ±0.1$^{c}$ |
| $A^-B^+D^+$ | 13.2 ±0.1$^{a}$ | 15.6 ±0.3$^{b}$ | 16.1 ±0.1$^{c}$ | 16.1 ±0.0$^{c}$ | 15.6 ±0.1$^{b}$ | 15.7 ±0.1$^{bc}$ | 15.9 ±0.0$^{bc}$ |
| $A^+B^-D^+$ | 13.2 ±0.0$^{a}$ | 13.4 ±0.0$^{a}$ | 16.2 ±0.1$^{cd}$ | 16.4 ±0.0$^{d}$ | 16.2 ±0.1$^{cd}$ | 15.8 ±0.1$^{b}$ | 15.9 ±0.1$^{bc}$ |
| $A^+B^+D^-$ | 15.9 ±0.1$^{ab}$ | 16.4 ±0.0$^{bc}$ | 16.5 ±0.4$^{c}$ | 16.1 ±0.1$^{abc}$ | 16.0 ±0.2$^{abc}$ | 15.9 ±0.0$^{ab}$ | 15.7 ±0.0$^{a}$ |
| $A^-B^-D^+$ | 15.2 ±0.1$^{a}$ | 16.0 ±0.1$^{bc}$ | 16.0 ±0.0$^{bc}$ | 16.1 ±0.0$^{c}$ | 15.8 ±0.1$^{b}$ | 15.8 ±0.1$^{bc}$ | 15.7 ±0.1$^{b}$ |
| $A^-B^+D^-$ | 15.6 ±0.0$^{ab}$ | 16.2 ±0.1$^{cde}$ | 16.5 ±0.1$^{e}$ | 16.3 ±0.1$^{de}$ | 16.1 ±0.0$^{cd}$ | 15.9 ±0.1$^{bc}$ | 15.5 ±0.1$^{a}$ |
| $A^+B^-D^-$ | 13.1 ± 0.1$^{a}$ | 15.6 ±0.2$^{b}$ | 16.2 ±0.2$^{d}$ | 16.0 ±0.1$^{cd}$ | 15.7 ±0.0$^{bc}$ | 15.5 ±0.0$^{b}$ | 15.3 ±0.1$^{b}$ |
| $A^-B^-D^-$ | 13.0 ±0.0$^{a}$ | 14.8 ±0.3$^{b}$ | 16.5 ±0.0$^{d}$ | 16.6 ±0.1$^{d}$ | 16.7 ±0.1$^{d}$ | 16.2 ±0.1$^{cd}$ | 16.0 ±0.1$^{c}$ |

[1]Values are means of three replications ± standard error. Means within the same row followed by different superscripts are significantly different ($p < 0.05$) with superscript 'a' being the lowest value. Average DP = $\Sigma$ ($DP_n$ x peak area) / $\Sigma$ (peak area)$_n$.

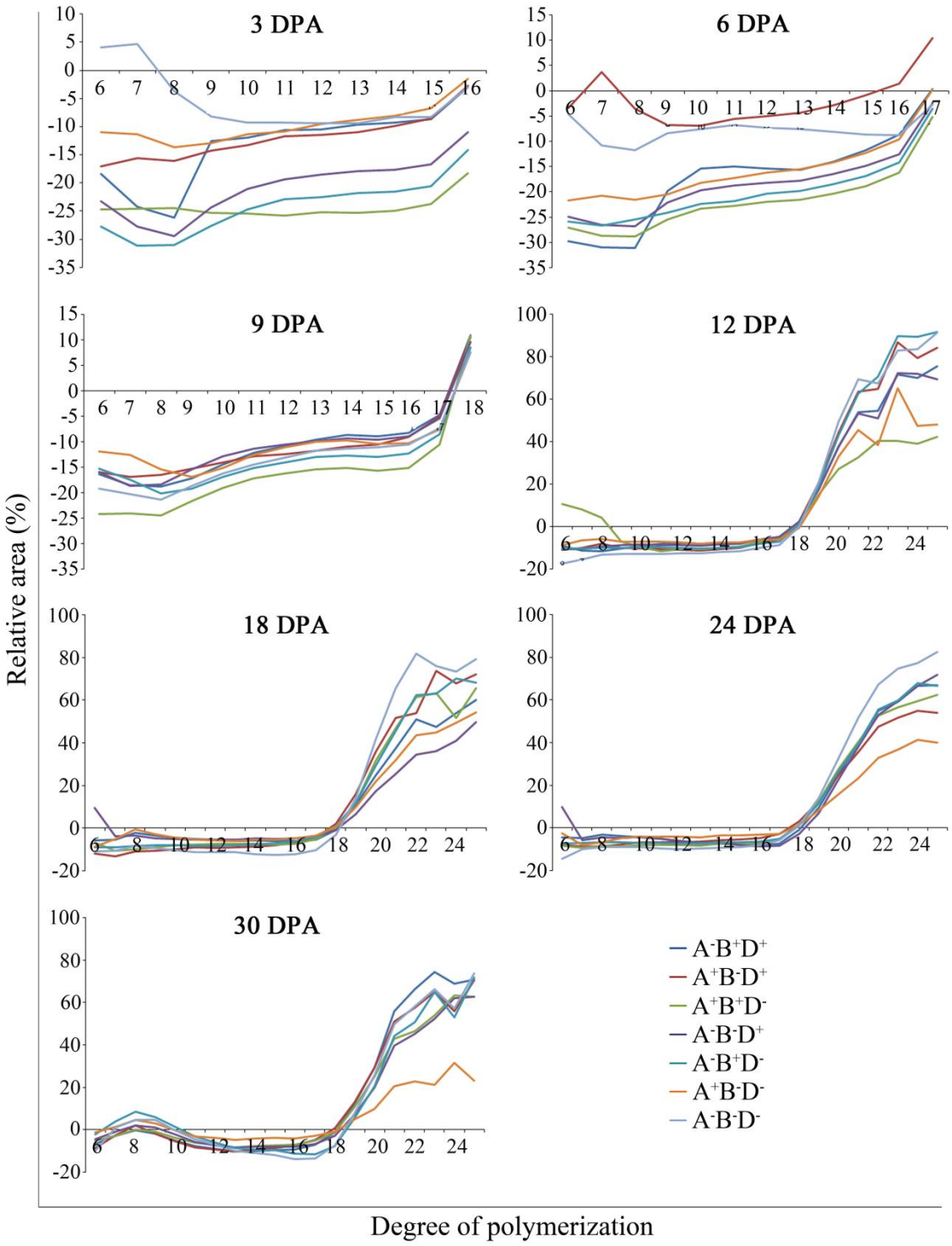



Figure 6.7 Amylopectin chain length distribution in partial and completely waxy wheat starch at 3, 6, 9, 12, 18, 24 and 30 DPAs

Subtractive graphs depict relative normalized obtained by subtracting non-waxy from waxy-null starches. Values plotted are based on means of three replications.

Table 6.2 Biopolymer fibril properties[1] of amylose and amylopectin from non-waxy and completely waxy starch at different development stages

| Sample | | Average height (nm) | Contour length | $M_n$ (x $10^5$Da) | $M_w$ (x $10^5$Da) | Polydispersity index ($M_w/M_n$) | Degree of polymerization ($M_n/MW_{monomer}$) |
|---|---|---|---|---|---|---|---|
| Non-waxy | 6 DPA | 1.6 ±0.4 | | | | | |
| | 12 DPA | 0.6 ±0.2 | 180 ±129 nm | 2.20 | 3.31 | 1.50 | 1222 |
| | 30 DPA | 0.6 ±0.3 | 180 ±164 nm | 2.23 | 4.03 | 1.81 | 1239 |
| Waxy | 6 DPA | ~ 3.6 | ~ 11 μm | | | | |
| | 12 DPA | ~ 1.4 | ~ 9 μm | | | | |
| | 30 DPA | ~ 4.8 | ~ 15 μm | | | | |

[1]Average height and contour lengths are values obtained from three technical replicates. $M_n$ - number molecular weight. $M_w$ – mass molecular weight.

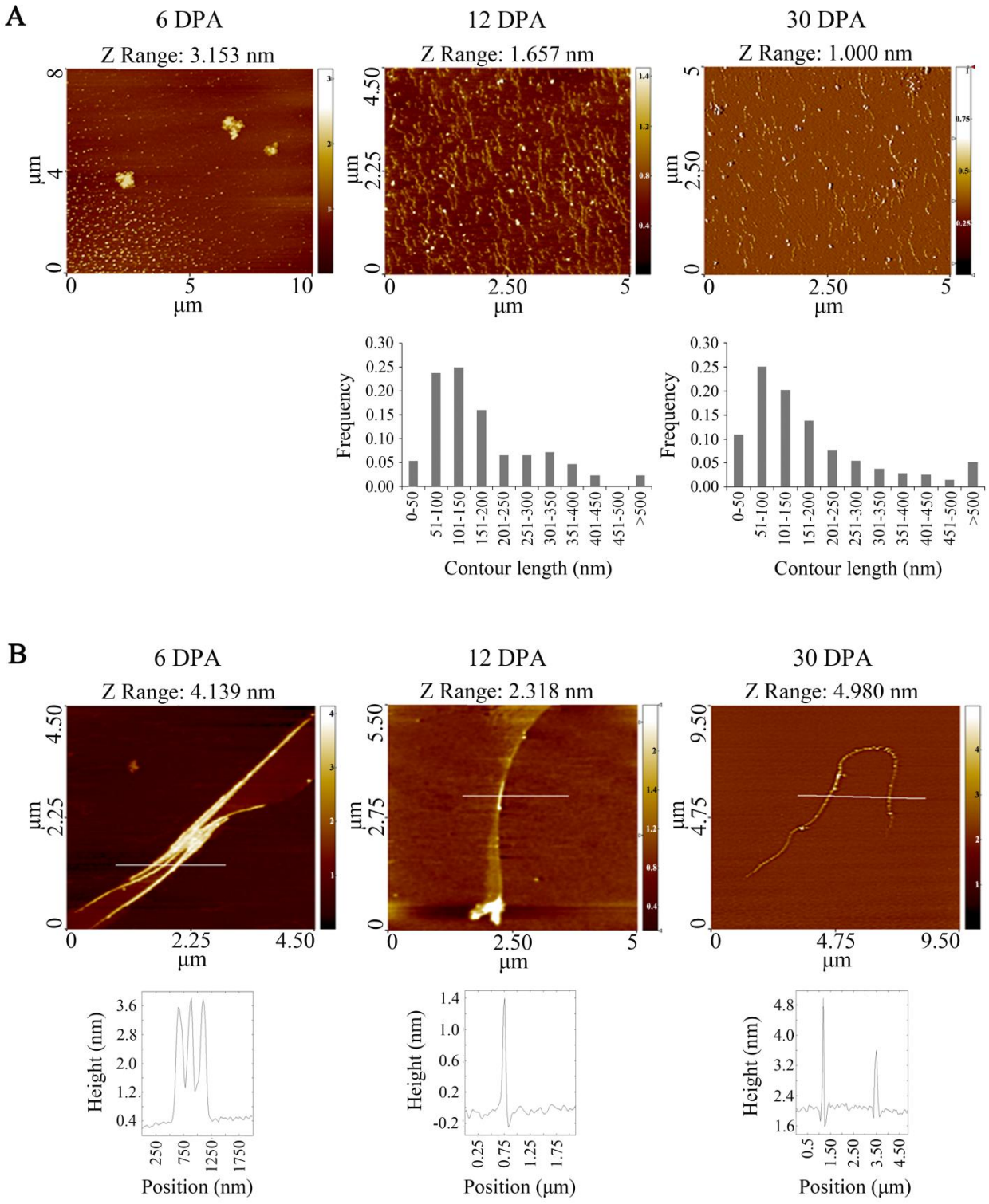



Figure 6.8 AFM images with corresponding contour length and height measurements at different development stages

(A) Non-waxy (B) Completely waxy starches

was less at 12 DPA, indicating more different kinds of structures (granules) at 30 DPA. In the non-waxy genotype, contour length frequency curve showed a bimodal distribution at 12 DPA, while unimodal at 30 DPA (Figure 6.8A). In the waxy genotype, amylopectin fibrils were observed at 6, 12 and 30 DPA (Figure 6.8B) with average heights of ~3.6 nm, ~1.4 nm and ~4.8 nm respectively (Table 6.2).

## 6.5 Discussion

Wheat endosperm consists of four cell types; the embryo surrounding cells, transfer cells, aleurone layer and the starchy endosperm (Olsen, 2001). Starchy endosperm in wheat represents the largest body of cell mass in the endosperm consisting of an estimated 60,000 cells (Chojecki *et al*., 1986). Development of endosperm is broadly divided into five phases (Simmonds and O`Brien, 1981). Phase I (0 DPA) is the fertilization phase. Phase II (1-5 DPA) is the grain growth stage where the initial endosperm nucleus divides repeatedly forming coenocytic stage endosperm. Phase III (6-13 DPA) is the initial grain filling stage when cellularization occurs via formation of radial microtubule systems and alveolation (Olsen, 2004). Phase IV (14-24 DPA) is the maximum grain filling stage in which rapid starch and protein synthesis occurs in the endosperm (Bechtel, 1990; Stoddard, 1999). Phase V (25-38 DPA) is the dry down stage when desiccation occurs. Morphological and biochemical changes occurring in starchy endosperm during wheat grain development will provide an insight into the factors affecting the final wheat grain quality.

### 6.5.1 Starch granules morphology changes

In wheat endosperm, starch granules are produced individually in separate amyloplasts. Starch granules of different shapes and sizes are developed in the endosperm during different periods of grain maturation, which have been divided into different classes. The number of size classes, however, is debated. All reports agree that in the initial grain development period, the first population of starch granules (large A-type, >15 μm) emerges at 4 – 7 days post anthesis (DPA) (Bechtel and Wilson, 2003). These are initially spherical granules of diameter 0.5 – 1.0 μm, which grow radially to 2 – 4 μm. According to Evers (1971), an equatorial plate is formed after a certain size is reached, which encircles the granule. Active deposition of glucan molecules takes place over the equatorial plate, giving the granule its characteristic lenticular shape. A

second population of small starch granules (B-type, 5-15 μm) is initiated at 12 – 14 DPA, which develop inside amyloplasts protrusions and remain spherical throughout development (Bechtel and Wilson, 2003). Some reports suggest the occurrence of third population of even smaller spherical starch granules (C-type, < 5 μm) at 21 DPA (Raeker *et al*., 1998).

In the present study, the first population of small spherical starch granules (possibly A-type) was observed at 3 and 6 DPA, which increased in size from 9 DPA onwards (Figure 6.1A, B). This indicates starch accumulation at the coenocytic stage, before endosperm cell wall formation (Simmonds and O'Brien, 1981). Also, expression of GBSSI in wheat at this stage has been previously reported (Laudencia-Chingcuanco *et al*., 2007; Nadaud *et al*., 2010), suggesting starch formation as early as 3 DPA. The equatorial groove for granule enlargement was observed from 9 to 15 DPA, after which the granules assumed a lenticular shape. A second population of starch granules (possibly B-type) was observed at 9 and 12 DPA. After 18 DPA, the burst of a third population of small starch granules (possibly C-type) was observed. At maturity, both large and small granules were present. Completely waxy genotype contained a higher proportion of small granules, supporting the higher rate of hydrolysis of waxy starch as compared to non-waxy or partial waxy starch.

### 6.5.2 Carbohydrate accumulation

Starch and amylose content showed a consistent increase with maturity, agreeing with previous reports for wheat (Kulp and Mattern, 1973; Ganeshan *et al*., 2010; Wei *et al*., 2010; Zhang *et al*., 2010), barley (McDonald *et al*., 1991), and maize (Li Li *et al*., 2007); however differing with potato where apparent amylose did not significantly change with maturity (Liu Q *et al*., 2003). A previous transcriptional profiling study in wheat suggested that the maximum rate of accumulation of transcripts encoding storage proteins and starch biosynthetic enzymes occurs from 7-14 DPA (Laudencia-Chingcuanco *et al*., 2007), with a maximum starch accumulation rate between 10-14 DPA which peaked at 35-38 DPA. Similarly in the present study, starch concentration increased ~10% from 12-15 DPA in all genotypes (Figure 6.2). In the endosperm development pathway, this period marks the beginning of the grain filling stage. A significantly higher concentration of starch was observed in the non-waxy genotype throughout grain filling. Previously, starch accumulation has been determined by simulating Richard's growth equation (Yang *et al*., 2004) and it was postulated that starch accumulation is determined

by the average starch accumulation rate, not active starch accumulation duration (Dai, 2010). A high starch accumulation rate and higher enzyme activities of sucrose synthase (SuSy), AGPase, SS, GBSS and SBE were reported for a cultivar with higher starch content (Dai, 2010), supporting higher total starch concentration in non-waxy genotype in the present study.

Amylose concentration showed a linear increase in non-waxy and partial waxy genotypes (Figure 6.3). It has been shown earlier that mRNA of rice *Gbss1* increased with the development of endosperm (Dian *et al*., 2005). Also, that increase in *Gbss1* expression is directly related to amylose accumulation, in maize endosperm (Li Li *et al*., 2007). In the present study, amylose concentration was highest in the non-waxy genotype (30.5%). In a previous study comparing wheat lines with differing starch contents, it was shown that GBSS activity was higher in the cultivar with higher starch content, indicating its stronger ability to synthesize amylose and amylopectin than the cultivar with low starch content during mid-late grain filling (Dai, 2010).

The completely waxy genotype should have shown a negligible concentration of amylose from earlier stages. However, a ~7% amylose concentration was observed at 3 and 6 DPA, which decreased thereafter (Figure 6.3). It is speculated that the detected amylose could be due to GBSSII from pericarp, which is still photosynthetic at that stage. GBSSII is responsible for amylose synthesis in non-storage tissues (Fujita *et al*., 1998; Vrinten and Nakamura, 2000); and since it is encoded by a separate gene, absence of GBSSI would not affect amylose accumulation by GBSSII.

### 6.5.3 Waxy protein accumulation

In the non-waxy genotype, reduced accumulation of the waxy protein was observed during early grain filling, which increased and remained consistent throughout mid and late grain filling periods (Figure 6.4). In previous studies as well, presence of GBSSI was reported at the beginning of wheat and rice endosperm formation which increased regularly during development (Hwang *et al*., 2005; Nadaud *et al*., 2010). In the completely waxy genotype, GBSSI accumulation was observed at 3 and 6 DPA (Figure 6.4), which was also reflected in the higher amylose concentration at these stages (Figure 6.3). It is speculated to be GBSSII, which is responsible for amylose synthesis in non-storage tissues such as pericarp.

### 6.5.4 Amylopectin chain length distributions

Significant changes were observed in the amylopectin glucan chain length distribution pattern, with the content of glucan short chains of DP ≤ 14 decreasing, long chains of DP ≥ 19 increasing (Figure 6.6), and average degree of polymerization increasing during early stages of grain development (Table 6.1). In a previous study of endosperm starch properties during maize grain development, it was reported that average amylopectin branch chain length increased from 10 DAP to 14 DAP and then decreased on 30 and 45 DAP (Li Li *et al*., 2007). It was suggested that the structure of endosperm starch was not synthesized consistently throughout grain development.

Comparing the non-waxy, partial waxy and completely waxy wheat starches in the present study, it was found that absence/ dosage of waxy protein subtly affected amylopectin structure formation during grain development (Figure 6.7). Compared to non-waxy starch, completely waxy starch at 3 DPA showed a comparatively higher proportion of glucan short chains of DP 6-8 (Figure 6.7), suggesting a relatively earlier synthesis of amylopectin chains in the former as compared to the latter, concurring with a previous report (Cao *et al*., 2012a). At 3 DPA, the influence of Wx-B, Wx-D alone as well as in combination was the highest, whereas, the influence of Wx-A was the lowest on DP 6-8 glucan chains. Similarly at 6 DPA, Wx-BD affected the glucan short chain fraction more than the other partial waxy starches. This suggests that Wx-B and Wx-D are playing a greater role in amylopectin synthesis during the initial stages of grain development. At 9 DPA, however, Wx-AB showed a higher proportion of DP 6-15 glucan chains followed by Wx-B and Wx-D. However, Wx-A alone was the least effective in influencing DP 6-8 chains; suggesting that Wx-A only in combination with Wx-B or Wx-D has a stronger effect on the glucan short chains formation. Furthermore, at 12 DPA, Wx-AB displayed a considerably higher proportion of short chains of DP 6-8 than the other partial waxy and non-waxy wheat starches, suggesting a positive influence of Wx-A on the short chain phenotype during grain filling. At 18 and 24 DPA, an increase in DP 6-8 glucan chains relative to the non-waxy starch was observed for Wx-D. In addition at 24 and 30 DPA, glucan chains of DP > 20 were negatively affected by Wx-A (Figure 6.7). This suggests that Wx-B and Wx-D play a stronger role in influencing mid-length glucan chains than Wx-A, during later stages of grain development. The above observations suggest that different waxy isoproteins exhibit different

capacities for amylopectin structure formation during wheat grain development, with Wx-B and Wx-D being the most effective during early grain development, Wx-A being active as grain filling starts, followed by Wx-D during grain maturation.

It has previously been reported that the relative expression of *Gbss1* increases only after 5 DPA (Ganeshan *et al*., 2010). However other starch synthases (*SS1, SS2*) show a higher relative expression than *Gbss1* at earlier DPA. For the non-waxy genotype, therefore, the reduction in the content of glucan short chains (Figure 6.6) could be a result of increased *Gbss1* expression. As *Gbss1* expression increases, it actively elongates the glucan short chains; hence the number of glucan short chains decreases and glucan long chains increases. In addition, 11 enzymes associated with starch synthesis, particularly GBSSI and soluble SS, have been shown to have expressed at the very beginning of wheat grain development (Nadaud *et al*., 2010), which could be the contributing factor for amylopectin synthesis in the absence of GBSSI.

The content of glucan short chains was higher during early endosperm development stage, mainly during the cellularization stage; which decreased consistently until the initial grain filling stage. From active grain filling onwards, the amylopectin chain length distribution changed only slightly (Figure 6.6, 6.7). It suggests that amylopectin attains its specific unit chain length distribution/ structure by the grain filling stage. Starch biosynthetic enzymes activity assays during endosperm development could provide a better understanding of amylopectin structure formation during grain development, as well as the precise function of GBSSI.

### 6.5.5 Starch component morphology changes

In the non-waxy genotype, at 6 DPA, only nanoparticles were observed (Figure 6.8A), which could reflect the aggregation of newly formed short chains of amylopectin during the initial cellularization phase. At 12 and 30 DPA, numerous small fibrils, some with branches, were observed, suggesting that they are amylopectin fibrils, as has been observed for potato (Dang *et al*., 2006). In a previous study, purified amylose and amylopectin were imaged under AFM and spider-like features for amylopectin were reported (Liu *et al*., 2001). Although such features were not observed in the present study, branching does indicate the presence of amylopectin. At 12 and 30 DPA, small unbranched fibrils were also observed, which could be amylose (Liu *et al*., 2001), as was previously observed in rice starch (Dang *et al*., 2006).

During imaging under non-contact mode of AFM, the contrast of the fibrils is related to their diameter (Liu *et al*., 2001). In other words, the greater the contrast between the fibril and the background, the thicker the fibril is. Compared to 12 DPA, fibrils at 30 DPA showed a greater contrast, suggesting a more folded conformation of the fibrils at maturity; by which time amylopectin has attained its final structure/ conformation.

In the waxy genotype, double helical amylopectin fibrils were observed throughout development (Figure 6.8B). Higher average height at 6 DPA could be explained by the conformation of coiled fibrils, which when unwound and in a single helical state had a low average height at 12 DPA. At 30 DPA, amylopectin could have assumed a different conformation, and may be super coiled, hence showing a high average height. Amylopectin fibrils could be retrograded in the waxy starch during sample preparation. The property of retrogradation has been utilized to image wheat starch, maize starch, and lipid-complexed amylose for utilization in food processing (Tang and Copeland, 2007).

A higher fibril average height was observed for waxy starch as compared to non-waxy starch at all stages of grain development; differing from a previous report in barley where starches with varying concentrations of amylose were imaged and higher fibril heights for non-waxy cultivars as compared to waxy starches were observed (Maley *et al*., 2010).

X-ray analysis suggests that amylose upon gelatinization adopts a "V" structure and the helix undergoes a net 30° rotation upon rehydration (French and Zaslow, 1972). Considering that six glucose residues are present per turn in the helix, the change to a "V" conformation would lead to 1.32 Å rise per residue. Per V-amylose chain, the linear mass density is ~1220 Da $nm^{-1}$ (French and Zaslow, 1972). Using these parameters, mass average molecular weight, number average molecular weight, polydispersity index (PDI) and degree of polymerization were determined (Table 6.2). PDI for non-waxy starch at 12 and 30 DPA were 1.50 and 1.81 respectively, which is in agreement with a previous report where synthetic amylose (PDI = 1.25) was imaged under AFM (McIntire and Brant, 1999).

Previously, it has been suggested that the amylopectin lamellar structure of barley imaged through AFM could complement the Side Chain Liquid Crystalline Polymeric (SCLCP) model (Waigh *et al*., 2000), with the fibril heights corresponding to flexible spacers and mesogen

moieties (Maley *et al*., 2010). Further extensive study is required for depiction of the amylopectin lamellar structure for wheat starch using AFM.

## 6.6 Conclusions

Endosperm development is a complex process involving numerous physiological and biochemical transitions. Granule morphology continuously changed with each phase of development; resulting in consistent starch and amylose accumulation in non-waxy and partial waxy genotypes. Changes in amylopectin structure were observed until 12 DPA, suggesting the formation of a basic amylopectin skeleton by the grain filling stage, which was also observed with AFM. Different waxy isoproteins affected amylopectin structure differently during wheat grain development. In the early stages, Wx-B and Wx-D were the most effective, followed by Wx-A during the mid grain-fill stage and Wx-D during grain maturation. Thus, genome specific GBSSI in wheat considerably affects starch accumulation and glucan chain length distribution during grain development.

# 7. GENERAL DISCUSSION AND CONCLUSIONS

## 7.1 Inferences

First study dealt with analyzing six natural Canadian wheat cultivars for their starch granule composition, structure and *in vitro* starch digestibility. These cultivars belong to two Canadian wheat classes- CWRS and CPSR, and included two CWRS cultivars (CDC Teal, AC Superb) with a complete complement of GBSSI, two CWRS cultivars (AC Barrie, AC Splendor) with GBSSI-B absent (Demeke *et al*., 2000) and two CPSR cultivars (AC Foremost, AC Crystal). In a wheat grain, starch is the major storage carbohydrate and a source of calories in the human diet and in animal feed. In the not too distant past, the focus of food production was to meet the calorific needs of the human diet. In some parts of the world where hunger and malnutrition is prevalent, meeting the daily calorific requirement is still a challenge. However, in the developed countries of North America, Europe and among affluent population in Asia and Africa, over-consumption of calories along with a changing lifestyle has resulted in obesity and associated ailments resulting in chronic diseases and poor health. Therefore, it is important to study the factors that determine starch digestibility so that the calorific production of food can be regulated to meet the requirements of individual consumers according to their needs. In this work, studies were conducted to understand wheat starch structure and factors that influence *in vitro* starch hydrolysis so that calorific production can be controlled.

In the plant kingdom, physical and chemical characteristics of starch vary with the storage organ. From a human digestion perspective, it is interesting to analyze how the same starch but derived from different botanical sources behaves. Numerous studies have been conducted on determining the rate of hydrolysis of starch extracted from different plants. Starch properties that affect starch enzymatic hydrolysis include starch granule size and shape (Vasanthan and Bhatty, 1996; Jane *et al*., 1997; Benmoussa *et al*., 2006), amylose-amylopectin ratio (Ito *et al*., 1999), amylopectin chain length distribution (Song and Jane, 2000), packing of amorphous and crystalline regions (Gallant *et al*., 1992; Zhang *et al*., 2006), crystallinity of the starch granule (Planchot *et al*., 1997), amylose-lipid complexes (Crowe *et al*., 2000), inherent enzyme inhibitors (Bjorck *et al*., 1987) and pores on starch granule surface. Some of these factors have been studied in this thesis and will be discussed.

Between the two wheat classes, AC Crystal from CPSR showed significantly ($p < 0.05$) higher total starch and amylose content as compared to other cultivars. Among the CWRS cultivars, GBSSI-B absence did not affect amylose accumulation, suggesting that genome-specific GBSSI might not be a limiting factor for amylose synthesis. Considering the buffering effect of genomes, GBSSI-A and GBSSI-D might be compensating for the lost GBSSI-B function. Amylose content is an important determinant of starch's hydrolysis potential. The two CPSR cultivars, AC Foremost and AC Crystal, with relatively increased amylose concentration, also displayed lower hydrolysis indices for both meal and extracted starch.

Starch structure or amylopectin chain length distribution was analyzed using FACE, which revealed differences between the two wheat classes. Although the average degree of polymerization did not show significant ($p < 0.05$) differences, considerable differences were observed in medium sized chains (DP 15-18) and long chains (DP 37-45) of amylopectin. CPSR cultivars showed reduced proportion of glucan chains of DP 15-18 and higher proportion of glucan chains of DP 37-45. As mentioned above, CPSR cultivars also had a reduced rate of starch hydrolysis. Previously, a parabolic relationship between amylopectin chain length distribution and *in vitro* starch digestibility in maize was put forward (Zhang *et al*., 2008). It was proposed that amylopectin with either a higher proportion of very short chains or very long chains, is relatively slowly digestible. In our results also, we observed that CPSR cultivars (AC Foremost and AC Crystal) possessed higher proportion of long chains of DP 37-45, had higher resistant starch content, hence suggesting the role of long chains to reduce digestibility of starch. In addition, reduced mid-length chains of amylopectin in rice have been associated with higher RS (Shu *et al*., 2007). Similarly, reduced proportion of DP 15-18 in the CPSR cultivars also supports the report in rice. Therefore, we suggest that these fractions of chains (reduced DP 15-18 and increased DP 37-45) might contribute towards reduced rate of starch *in vitro* enzymatic hydrolysis in CPSR cultivars.

Various studies have been conducted on studying the unit chain length distribution of amylopectin in different plant systems (Li *et al.*, 2001; Tang, 2002; Yoshimoto *et al.*, 2002; Singh *et al.*, 2009). Substantial differences in the structure of amylopectin from different botanical sources have been reported (Hanashiro *et al*., 1996; Jane *et al*., 1999; Silverio *et al*., 2000; Srichuwong *et al*., 2005). Although fine structure of amylopectin has been studied using different methodologies such as High-performance size-exclusion chromatography (HPSEC)

with differential refractive index detector (Hizukuri, 1985; 1986; Reddy *et al*., 1993), High performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) (Koizumi *et al*., 1991), Fluorophore-assisted capillary electrophoresis (FACE) (Srichuwong *et al*., 2005); all of these different distributions are consistent with the cluster model of amylopectin architecture. Amylopectin chain length distribution of different cereal and tuber starches using HPAEC-PAD has been studied (Hanashiro *et al.*, 1996). Starches from different origins displayed variable peaks and shoulders in the glucan chain length distribution curve. Peak DP of rice, wheat, barley, maize, sweet potato, yam and lotus was 12, 11, 11, 13, 13, 14 and 13 respectively. Shoulder DP of the same plants was 18, 18, 18, 19, 20, 20 and 18 respectively. Similar variation was observed in amylopectin chain length distribution, especially short chains, in different plant systems (Jane *et al*., 1999). Cereal starches displayed an increased proportion of DP 7-9 chains. On the other hand, tuber, root and legume starches displayed higher proportion of DP 7 than DP 6 or 8 chains. Both the studies mentioned above showed a shoulder at DP 18-21, however the relative intensity of the shoulder varied between species. Differences in amylopectin chain length distribution and relative intensities were reported even within cereals themselves. In an amylopectin retrogradation study, it was suggested that cereal amylopectins like wheat, barley and rye, except maize, showed very similar distribution profiles (Silverio *et al.*, 2000). Wheat, barley, amaranth and tapioca possessed prominent shoulders, while it was absent in maize, rice, millet, Chinese taro and green banana (Jane *et al*., 1999). It can be concluded from here, that amylopectin structure is species specific.

Since short chains of amylopectin tend to generate crystallinity in a starch granule; variations in amylopectin chain length distributions would therefore affect crystallinity. It has been reported previously that crystallinity affects digestibility of starch, with the A-type crystalline starches (cereals) being more susceptible to hydrolysis than the B-type crystalline starches (tubers) (Valetudie *et al.*, 1993; Planchot *et al*., 1997; Jane, 2007). It was found that the hydrolysis degree of B-type crystalline starches did not change with increased incubation time with amylases (Srichuwong *et al*., 2005). Previous reports have shown that amylopectin of A-type crystalline starch contains a higher proportion of shorter branched chains (Hizukuri *et al.*, 1983) and a higher number of chains per cluster (Takeda and Hanashiro, 2003) than B-type crystalline starch, which could explain the higher relative digestibility of the former. It can thus

be said, that composition and physical properties of starch granules affect their enzymatic hydrolysis properties, which ultimately determine their calorific contribution in human diet.

Besides starch composition and structure, starch granule size distribution has also been shown to affect the rate of starch digestibility. Few reports have mentioned that higher proportion of small starch granules result in faster hydrolysis of starch (Salman *et al*., 2009; Teo and Small, 2012). A CWRS cultivar, AC Splendor showed reduced volume of C-type starch granules (~25%), and increased volume of A-type starch granules (~5%) than other CWRS cultivars. In addition, it also possessed lowest hydrolysis index (126) among the CWRS cultivars. Thus, higher proportion of large starch granules and low proportion of small starch granules could reduce the rate of starch *in vitro* hydrolysis in wheat.

Starch granule size and shape varies in *Chlamydomonas*, cereals, pulses and tubers, being as small as 0.5-2.5 μm lenticular flat in *Chlamydomonas* (Buléon *et al*., 1997) and oval ~100 μm in potato (Hoover, 2001). Starch granule size distribution even varies within cereals. For example, in rice, oats and maize, starch granule sizes vary from 2 to 10, 14 and 30 μm respectively (Takeda *et al*., 1990). In wheat, barley, triticale and rye, starch granules have been sub-divided into different classes based on their sizes. Small starch granules in these cereals are spherical, while the large granules are lenticular in shape. Overall, their sizes range from 1 μm to 50 μm (Evers, 1973; Jane *et al*., 1994; Tang *et al*., 2001). Starch granule size and shape directly affects its rate of hydrolysis. *In vitro* digestibility of starches from different botanical sources was studied, and the rate of hydrolysis of starches in 72 hr of incubation with amylases decreased with increasing starch granule size, which followed the order: rice (4.7 μm) > yam bean (7.7 μm) > cassava (23.2 μm) > potato (42.3 μm) (Srichuwong *et al*., 2005).

Therefore in addition to differences in starch and amylose concentrations in these cultivars (Hucl and Chibbar, 1996), amylopectin chain length distribution and starch granule size distribution also varied between the two classes, which influenced *in vitro* hydrolysis potential. CPSR cultivars with increased amylopectin chains of DP 37-45, reduced chains of DP 15-18, low volume of small starch granules, showed subtle effects on reducing the *in vitro* starch hydrolysis rate. More detailed analysis could establish the precise effect of starch structure on wheat starch digestibility.

Despite the difference in reserve organs, starch synthesis machinery is similar in all plant systems. Starch biosynthesis is a complex process, involving a number of enzymes some of which are present in multiple isoforms, that catalyze various steps which results in starch granules that are species specific (Smith *et al*., 1997; James *et al*., 2003). Wheat is an allohexaploid which contains three distinct genomes thus representing three copies of every gene. Although this complicates the study of the biosynthetic pathway in wheat, but it also provides an opportunity to study the dosage effects of a gene in the biosynthetic pathway. In starch biosynthesis, granule-bound starch synthase I (GBSSI) is the key enzyme responsible for amylose synthesis and recently it has been suggested to participate in amylopectin synthesis by synthesizing long glucan chains in *Chlamydomonas reinhardtii* (Ral *et al.*, 2006), wheat (Yoo and Jane, 2002), rice (Aoki *et al*., 2006) and potato (McPherson and Jane, 1999). In the present study, wheat genotypes deficient in GBSSI from A, B or D genomes singly or in combination were used to study their influence on starch granule composition and structure and their influence on *in vitro* enzymatic hydrolysis of starch.

Initial study was based on wheat cultivars being used commercially. Leading forward, synthetic wheats with GBSSI isoproteins deficient from one, two or three wheat genomes were studied to analyze its effect on starch structure, composition and *in vitro* enzymatic hydrolysis potential.

Second part of this thesis focussed on the relationship between *in vitro* starch enzymatic hydrolysis and starch characteristics in single, double and triple waxy-null near-isogenic lines i.e., Wx-A$^-$, Wx-B$^-$, Wx-D$^-$, Wx-A$^-$B$^-$, Wx-A$^-$D$^-$, Wx-B$^-$D$^-$, and Wx-A$^-$B$^-$D$^-$. Amylose synthesis capacity is variable between the three waxy proteins from each genome (Miura *et al*., 1999; Debiton *et al*., 2010), and different combinations of waxy proteins result in different concentrations of starch constituents (Demeke *et al*., 1999; Kim *et al*., 2003). Additionally, absence of GBSSI alters amylopectin chain length distribution (Li Chun-Yan *et al*., 2007). Thus, we had hypothesized that absence of one or more waxy proteins would differently affect starch composition, structure and hydrolysis properties.

Waxy proteins from different genomes of wheat have been postulated to affect the amylose concentration differently (Miura and Sugawara, 1996). In this study, amylose concentration was affected more severely when waxy proteins from two genomes were missing (double nulls) as

compared to when waxy protein from one genome was missing (single nulls), suggesting a dose dependent effect of waxy protein on amylose accumulation, concurring with previous reports (Demeke *et al*., 1999; Kim *et al*., 2003). Although gradual reduction of amylose concentration with the loss of each waxy protein was recorded; however no drastic effects were observed. This could be attributed to the compensatory function of the remaining waxy proteins. It is also implicated that amylose content was not linearly proportional to the number of *Wx* genes, suggesting that the *Wx* genes act in an epistatic manner (Miura *et al*., 1999). Additionally, the order of amylose synthesis capacity has been suggested to be as Wx-B1 ≥ Wx-D1 > Wx- A1 (Miura *et al*., 2002) and/or Wx-D1 > Wx-B1 > Wx-A1 (Debiton *et al*., 2010). However, we did not observe any specific order for GBSSI deficiency on amylose synthesis.

Waxy protein has been shown to influence the long chain fraction of amylopectin (Ral *et al*., 2006). Alternatively a study in wheat showed that starch synthase and starch branching enzymes are not regulated by the *Wx* null alleles (Debiton *et al*., 2010), suggesting that amylopectin formation pathway is not affected by lack of one, two or three *Wx* alleles. In addition, it has been shown that amylopectin structure is identical in waxy and their respective parents in wheat and rice near-isogenic lines (Yasui *et al*., 1996; Zhao and Sharp, 1998; Miura *et al*., 2002). However, in the present study, minor but significant ($p < 0.05$) differences were observed in the very short chain fraction, with Wx-$A^+B^+D^+$ showing higher concentration of DP 6-8 chains than Wx-$A^-B^-D^-$. Among the partial waxy genotypes, Wx-A and Wx-B has been postulated to have an additive contribution, and Wx-D has been suggested to have a suppressive role for short chain phenotype of amylopectin. Because only subtle differences in amylopectin chain length distribution were observed in non-waxy, partial and completely waxy starches, we can propose that GBSSI is not limiting for amylopectin synthesis.

Granule size is an important characteristic that influences composition of starch and its functionality. It has been shown previously, that large starch granules possessed greater amylose content than small starch granules, while the latter possessed higher proportion of short amylopectin chains (Kim and Huber, 2010). In the present study, the completely waxy starch showed relatively higher volume percentage of small C-type starch granules (~38%) and lower volume percentage of large A-type starch granules (~10%), compared to non-waxy and partial waxy starch, concurring with previous reports in wheat (Bertolini *et al*., 2003) and barley (Asare *et al*., 2011).

All the above mentioned starch characteristics affected starch *in vitro* enzymatic hydrolysis properties. It has been shown earlier that higher amylose concentration reduces starch digestibility (Frei *et al.*, 2003; Chung *et al.*, 2010; Zhu *et al.*, 2011). The reason for such characteristic lies in the linear structure of amylose. Straight chains of amylose tend to form stable double helices, which are difficult to denature, and hence reduce the rate of hydrolysis (Lehmann and Robin, 2007). In agreement with these reports, the completely waxy genotypes, which lacked amylose, showed highest rate of *in vitro* starch enzymatic hydrolysis. Pearson's bivariate correlation analysis showed a negative correlation of amylose with hydrolysis index ($r = -0.77$, $p < 0.01$) and RDS ($r = -0.61$, $p < 0.01$); and a positive correlation with RS ($r = 0.49$, $p < 0.01$). Rate of hydrolysis is affected by the crystallinity of starch as well. Increased amylose starch shows B-type crystallinity, instead of normal A-type crystallinity of wheat starch; hence making it more resistant to hydrolysis (Ao *et al.*, 2007). In addition to amylose content and amylopectin chain length distribution, granule size distribution has also been shown to influence rate of *in vitro* starch hydrolysis. Larger size, lenticular shape, higher amylose (Peng *et al.*, 1999) and long chains of amylopectin (Ao and Jane, 2007) in A-type starch granules are the reasons for their reduced digestibility. In the present study, large A-type starch granules were negatively correlated with hydrolysis index ($r = -0.46$, $p < 0.01$) and small C-type starch granules were positively correlated with hydrolysis index ($r = 0.50$, $p < 0.01$) and negatively correlated with SDS ($r = -0.36$, $p < 0.05$). Additionally, the rate of hydrolysis was considerably slower in meal samples than extracted starch. This could be because of the presence of intrinsic factors in meal such as α-amylase inhibitors, β-glucan, lipids etc., which are absent in extracted starch.

Among the waxy-null genotypes, Wx-D had a suppressive role in the formation of short chain fraction of amylopectin. In addition, presence of Wx-D led to increased volume percent of large A-type starch granules (~9%) than non-waxy, partial waxy and completely waxy genotypes. Moreover, individual Wx-D showed significantly ($p < 0.05$) lower hydrolysis index (115.8), while it was higher when Wx-D was present with Wx-A (133.7) or Wx-B (130.9). Therefore, it is suggested that among the Wx isoproteins, Wx-D might be the major contributor influencing starch hydrolysis in wheat. Hence, interplay of genome specific GBSSI proteins affects the overall starch digestion rate, with subtle differences in amylopectin chain length distribution (Figure 7.1).

In this thesis, differences in starch composition and structure were studied on mature grains of near-isogenic waxy-null lines of wheat. The third part of the thesis dealt with the influence of genome-specific GBSSI on starch characteristics during wheat grain development.

Starch granules of different shapes and sizes are formed at different times during wheat grain development. Also, shape of a granule changes with development. Hence, the hypothesis was proposed, that change in morphology could be accompanied by change in arrangement of glucan chains within the granule. Since, in addition to its role in amylose synthesis, GBSSI has been shown to be involved in amylopectin synthesis (Yoo and Jane, 2002), we studied the changes in arrangement of glucan chains during development in waxy-null lines. This would indicate if lack of a single/ double/ triple GBSSI affects the glucan chain length distribution during granule development. The study was conducted with pure starch isolated from wheat grains at 3-30 days post anthesis (DPA), with three day intervals.

Changes in starch granule morphology were observed using scanning electron microscopy. Different classes of granules display different shapes and sizes. First population of granules at 3 and 6 DPA was observed which increased in size from 9 DPA onwards along with the formation of an equatorial groove. Similarly, second and third population of granules were observed starting 12 and 18 DPA respectively (Bechtel *et al*., 1990; Bechtel and Wilson, 2003). Although the pattern of granule size distribution was similar during development for non-waxy and waxy genotypes, the waxy genotype showed higher number of smaller granules than the non-waxy at maturity. Higher proportion of small granules might be a factor responsible for higher rate of *in vitro* starch hydrolysis of waxy starch as compared with non-waxy starch, as also observed in the second study.

Carbohydrate accumulation rates varied with development, with total starch and amylose concentration being higher in single nulls than in double nulls, suggesting a significant influence of GBSSI dosage on starch accumulation during wheat grain development. From 3 to 6 DPA, starch concentration in genotypes with missing Wx-A doubled, while a small increase was observed in other genotypes. This suggests that Wx-A could be playing a determining role in starch accumulation. Also in previous reports, it has been suggested that the catalytic activity of

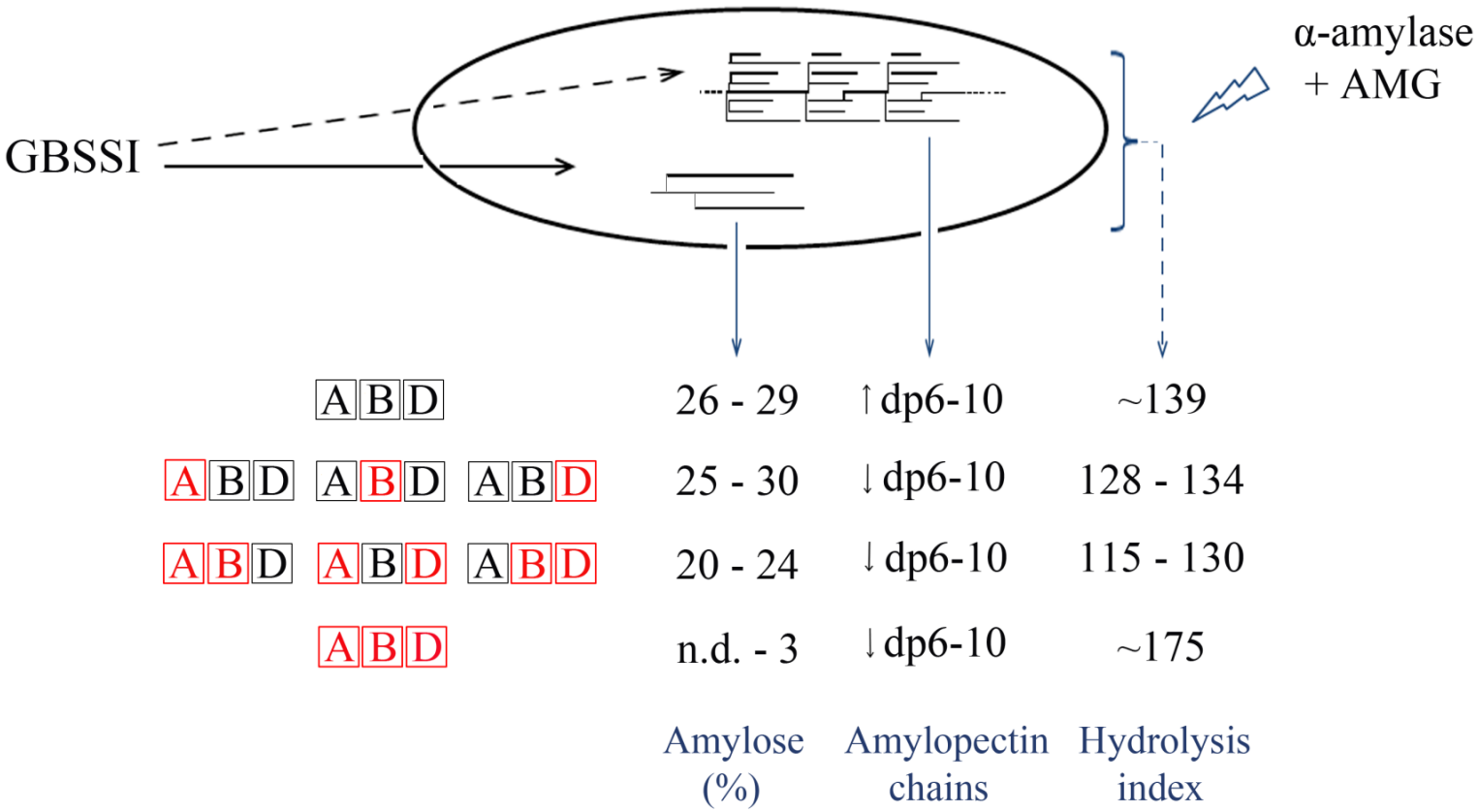



Figure 7.1 Effect of subtractive GBSSI dosage on amylose concentration, changes in amylopectin chain length distribution, and hydrolysis index

A, B, and D represent genome specific GBSSI in hexaploid wheat. Letters in red indicate the absence of that isoprotein. AMG – amyloglucosidase.

Wx-A is the lowest of the three isoforms of GBSSI (Miura *et al*., 1999; Debiton *et al*., 2010). This could be a supporting evidence for the same.

Significant changes were observed in the amylopectin chain length distribution pattern at different stages of development, as was also observed in maize (Li Li *et al.*, 2007). Most variation was observed from 3 to 12 DPA, after which there were no considerable differences in chain length distribution till 30 DPA. Chains of DP < 14 decreased significantly from 3 to 9 DPA; while chains of DP > 19 increased from 3 to 12 DPA. Chains of DP 15-18 did not show considerable differences during development. These results were also reflected in the increase in average DP from 3 DPA (12.3) to 9 DPA (14.7) and no change thereafter (15.0 at 30 DPA). Changes in amylopectin chain length distribution during development suggest that there is a continuous change in the length and arrangement of glucan chains up till at least 12 DPA, after which granules attain a specific shape, hence the average DP values do not significantly differ (Figure 7.2).

Differential effect of waxy isoproteins was observed in amylopectin structure formation during wheat grain development. Wx-B and Wx-D were postulated to play a greater role in amylopectin synthesis during initial stages of grain development. Positive influence of Wx-A was observed on short chain phenotype (DP 6-8) during the grain filling stage, and Wx-D showed maximum influence on mid-length chains (DP 19-22) during later stages of grain development (Figure 7.3).

In conclusion, subtle differences were observed in the amylopectin chain length distribution. Amylopectin chain length differences were associated with starch digestibility *in vitro*. CPSR cultivars (AC Foremost and AC Crystal) had a higher proportion of long chains of DP 37-45, reduced proportion of mid-length chains of DP 15-18, and a lower *in vitro* enzymatic hydrolysis potential. Absence of GBSSI revealed minor differences in the amylopectin chain length distribution. Factors such as amylose (negatively), amylopectin long chains (negatively) and small C-type starch granules (positively) influenced starch hydrolysis rate. Since starch components are important determinants of starch digestibility, amylopectin being a major component, further study on amylopectin chain length distribution was conducted at different stages of wheat grain development. Chains of DP < 14 reduced significantly, while chains of DP > 19 increased from 3-12 DPA, suggesting a continuous rearrangement of glucan chains till 12

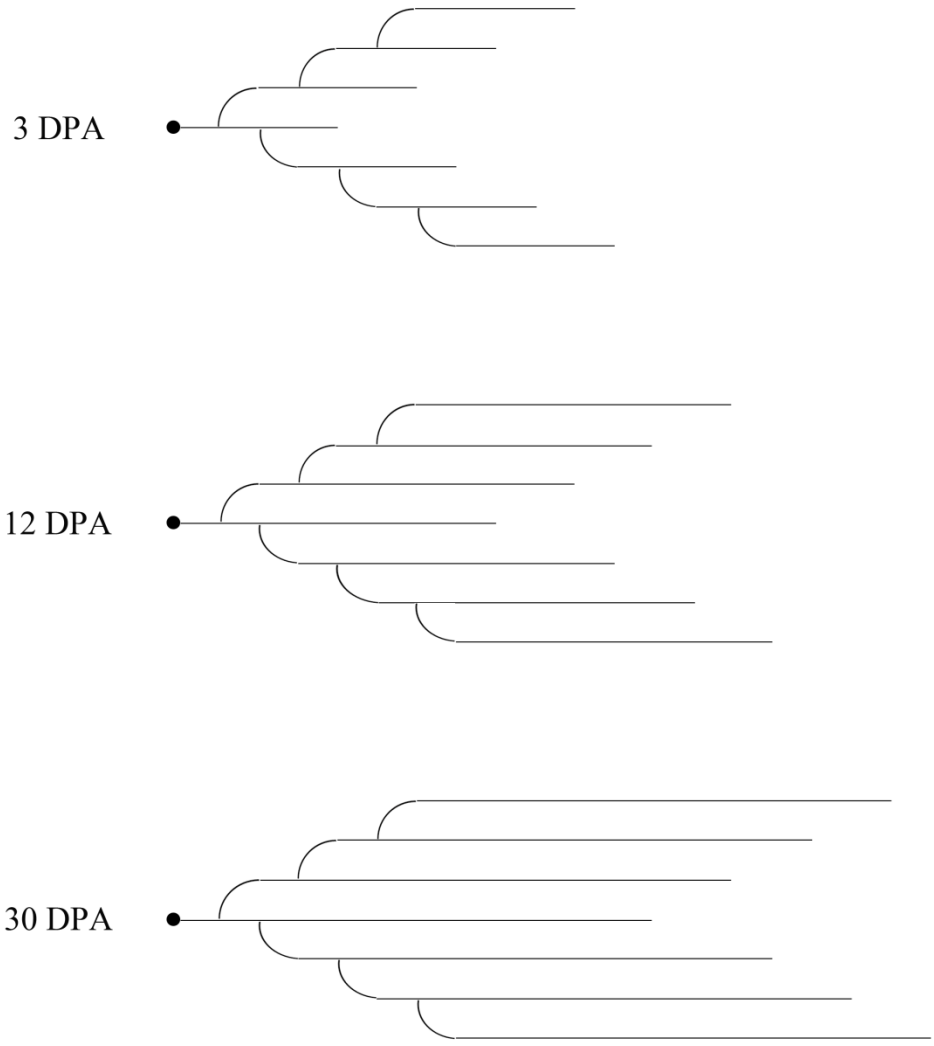



Figure 7.2 Overview of amylopectin chain length distribution during wheat grain development

Proportion of short chains is higher at 3 DPA, increases until 12 DPA, after which no significant changes occur in average DP at later stages.

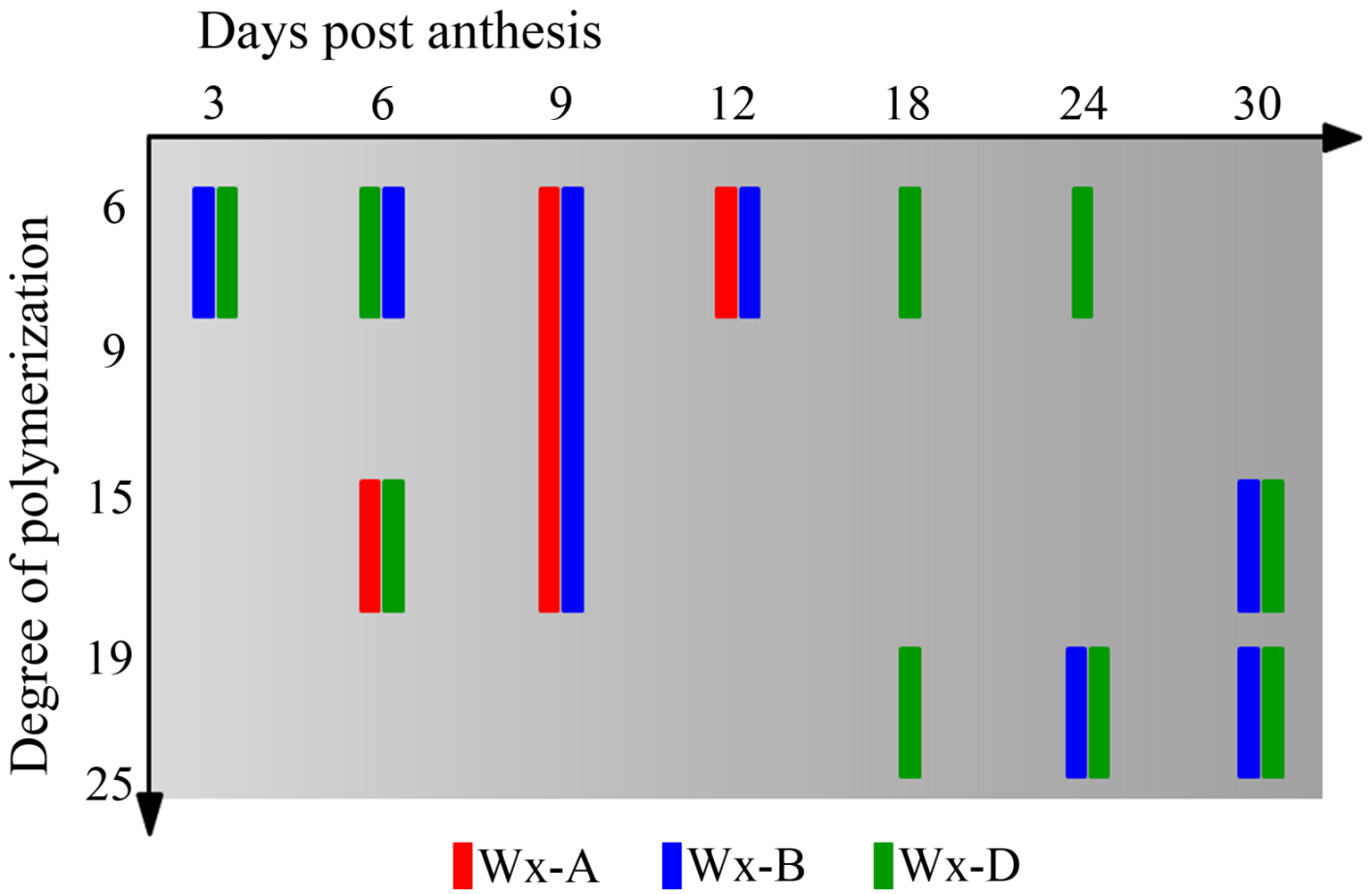



Figure 7.3 Influence of genome specific GBSSI on amylopectin structure formation during wheat grain development

Colored bars indicate higher activity of the respective isoprotein at a particular growth stage.

DPA. Also, waxy isoproteins differently affected the amylopectin structure formation. Major novel findings are listed below.

## 7.2 Novel findings

- Amylopectin chain length distribution association with starch *in vitro* enzymatic hydrolysis: Higher proportion of long chains of DP 37-45 in CPSR cultivars led to slowly digestible starch.
- Each GBSSI protein, by itself, is non-limiting for amylose synthesis: An absence of one GBSSI isoprotein did not affect amylose concentration as severely as the absence of two and/or three GBSSI isoproteins. An interaction between the remaining two GBSSI proteins in single nulls could be compensating for the missing protein's activity.
- Rate of starch *in vitro* enzymatic hydrolysis is affected by grain constituents: Grain proteins (such as α-amylase inhibitors), lipids, and β-glucan present in the grain meal reduced the rate of starch digestion as compared to pure starch, where these factors were absent.
- Amylopectin structure synthesis is completed by 12 DPA: Proportion of short chains decreased continuously till 12 DPA, after which the amylopectin chain length distribution remained unchanged, suggesting that basic architecture of amylopectin is determined by 12 DPA.
- Differential effects of GBSSI: In mature waxy-null lines, absence of waxy isoproteins affected the rate of starch *in vitro* enzymatic hydrolysis differently, by influencing starch composition and structure differently. During grain development, different waxy isoproteins influenced amylopectin structure at different stages.

## 7.3 Future research directions

This study has revealed the influence of genome specific GBSSI on starch composition, structure and *in vitro* hydrolysis properties. Some other aspects could be considered to obtain a better understanding of the mode of action of GBSSI towards starch granule formation:

- Monitoring the gene expression of starch biosynthetic enzymes during wheat grain development would indicate whether the absence of GBSSI from A, B and D genomes

has any pleiotropic effects on starch synthases, starch branching and debranching enzymes.

- Mode of action of GBSSI could also be a possible area of research, considering starch biosynthetic enzymes act in multi-protein complexes.
- Studying amylopectin chain length distribution at different time points during *in vitro* starch hydrolysis assay would represent specific changes in starch structure during digestion.
- Studying X- ray diffraction patterns would elucidate the changes in crystallinity during wheat grain development.
- Information about glucosidic bonds positioning and symmetry in starch could be obtained using monochromatic light scattering technique such as Raman spectroscopy.

# 8. LITERATURE CITED


AACC International. “Approved Methods of the AACC”. Methods 76-13; 46-30; 30-25; 32-23; 32-40. St Paul MN USA.

Abd Allah, M., Foda Y., Mahmoud, R., & Abouarab, A. (1987). X-ray-diffraction of starches isolated from yellow corn, sorghum, sordan and pearl-millet. *Starch/Stärke, 39*, 40-42.

Abdel-Aal, E.-S.M., Hucl, P., Chibbar, R. N., Han, H. L., & Demeke, T. (2002). Physicochemical and structural characteristics of flours and starches from waxy and nonwaxy wheats. *Cereal Chemistry, 79*, 458-464.

Ainsworth, C., Tarvis, M., & Clark, J. (1993). Isolation and analysis of a cDNA clone encoding the small-subunit of ADP-glucose pyrophosphorylase from wheat. *Plant Molecular Biology, 23*, 23-33.

Akman, Z., & Kara, B. (2003). Genotypic variations for mineral content at different growth stages in wheat (*Triticum aestivum* L.). *Cereal Research Communications, 31*, 459-466.

Albertsson, A., & Karlsson, S. (1995). Degradable polymers for the future. *Acta Polymerica, 46*, 114-123.

Ali, A. E., & AlArifi, A. (2009). Characterization and *in vitro* evaluation of starch based hydrogels as carriers for colon specific drug delivery systems. *Carbohydrate Polymers, 78*, 725 730.

Annison, G., & Topping, D. (1994). Nutritional role of resistant starch - chemical structure vs physiological-function. *Annual Review of Nutrition, 14*, 297-320.

Antonie, C., Peyron, S., Lullien, P. V., Abecassis, J., & Rouau, X. (2004). Wheat bran tissue fractionation using biochemical markers. *Journal of Cereal Science, 39*, 387-393.

Ao, Z., & Jane, J. (2007). Characterization and modeling of the A- and B-granule starches of wheat, triticale, and barley. *Carbohydrate Polymers, 67*, 46-55.

Ao, Z., Simsek, S., Zhang, G., Venkatachalam, M., Reuhs, B. L., & Hamaker, B. R. (2007). Starch with a slow digestion property produced by altering its chain length, branch density, and crystalline structure. *Journal of Agricultural and Food Chemistry, 55*, 4540-4547.

Aoki, N., Umemoto, T., Yoshida, S., Ishii, T., Kamijima, O., Matsukura, U. & Inouchi, N. (2006). Genetic analysis of long chain synthesis in rice amylopectin. *Euphytica, 151*, 225-234.

Asare, E. K., Jaiswal, S., Maley, J., Båga, M., Sammynaiken, R., Rossnagel, B. G., & Chibbar, R. N. (2011). Barley grain constituents, starch composition, and structure affect starch *in vitro* enzymatic hydrolysis. *Journal of Agricultural and Food Chemistry, 59*, 4743-4754.

Baba, T., Yoshii, M. & Kainuma, K. (1987). Acceptor molecule of granular-bound starch synthase from sweet-potato roots. *Starch/Stärke, 39*, 52–56.

Bae, J. M., Giroux, M., & Hannah, L. C. (1990). Cloning and characterization of the *brittle- 2* gene of maize. *Maydica, 35*, 317-322.

Båga, M., Glaze, S., Mallard, C. S. & Chibbar, R. N. (1999). A starch branching enzyme gene in wheat produces alternatively spliced transcripts. *Plant Molecular Biology, 40,* 1019-1030.

Båga, M., Repellin, A., Demeke, T., Caswell, K., Leung, N., Abdel-Aal, E.,Hucl, P., & Chibbar, R. N. (1999). Wheat starch modification through biotechnology. *Starch/Stärke, 51*, 111-116.

Båga, M., Nair, R. B., Repellin, A., Scoles, G. J., & Chibbar, R. N. (2000). Isolation of a cDNA encoding a granule-bound 152-kilodalton starch-branching enzyme in wheat. *Plant Physiology, 124*, 253-263.

Bálint, A., Kovacs, G., Erdei, L., & Sutka, J. (2001). Comparison of the Cu, Zn, Fe, Ca and Mg contents of the grains of wild, ancient and cultivated wheat species. *Cereal Research Communications, 29*, 375-382.

Ball, S., Marianne, T., Dirick, L., Fresnoy, M., Delrue, B., & Decq, A. (1991). A *Chlamydomonas reinhardtii* low-starch mutant is defective for 3-phosphoglycerate activation and orthophosphate inhibition of ADP-glucose pyrophosphorylase. *Planta, 185*, 17-26.

Ball, S., Guan, H., James, M., Myers, A., Keeling, P., Mouille, G., Buléon, A., Colonna, P., & Preiss, J. (1996). From glycogen to amylopectin: A model for the biogenesis of the plant starch granule. *Cell, 86*, 349 352.

Ball, S., Van de Wal, M., & Visser, R. (1998). Progress in understanding the biosynthesis of amylose. *Trends in Plant Science, 3*, 462-467.

Ballicora, M. A., Frueauf, J. B., Fu, Y. B., Schurmann, P., & Preiss, J. (2000). Activation of the potato tuber ADP-glucose pyrophosphorylase by thioredoxin. *Journal of Biological Chemistry, 275*, 1315-1320.

Beatty, M., Rahman, A., Cao, H., Woodman, W., Lee, M., Myers, A., & James, M. (1999). Purification and molecular genetic characterization of ZPU1, a pullulanase- type starch debranching enzyme from maize. *Plant Physiology, 119*, 255-266.

Bechtel, D., Zayas, I., Kaleikau, L., & Pomeranz, Y. (1990). Size-distribution of wheat-starch granules during endosperm development. *Cereal Chemistry, 67*, 59-63.

Bechtel, D., Zayas, I., Dempster, R. & Wilson, J. D. (1993). Size-distribution of starch granules isolated from hard red winter and soft red winter wheat. *Cereal Chemistry, 70*, 238-240.

Bechtel, D. B., & Wilson, J. D. (2003). Amyloplast formation and starch granule development in hard red winter wheat. *Cereal Chemistry, 80*, 175-183.

Bechtel, D. B., Abecassis, J., Shewry, P. R., & Evers, A. D. (2009). Development, Structure and Mechanical Properties of the Wheat Grain. In K. Khan & P. R. Shewry (Eds.), *Wheat Chemistry and Technology* (pp. 51-96). Minnesota: AACC International Press.

Bello-Pérez, L., Paredes-López, O., Roger, P., & Colonna, P. (1996). Amylopectin – properties and fine structure. *Food Chemistry, 56*, 171-176.

Benmoussa, M., Suhendra, B., Aboubacar, A., & Hamaker, B. (2006). Distinctive sorghum starch granule morphologies appear to improve raw starch digestibility. *Starch/Stärke, 58*, 92-99.

Benmoussa, M., Moldenhauer, K. A. K., & Hamaker, B. R. (2007). Rice amylopectin fine structure variability affects starch digestion properties. *Journal of Agricultural and Food Chemistry, 55*, 1475-1479.

Berry, C. S. (1986). Resistant Starch. Formation and measurement of starch that survives exhaustive digestion with amylolytic enzymes during the determination of dietary fiber. *Journal of Cereal Science, 4*, 301-314.

Bertoft, E. (1991). Chains of intermediate lengths in waxy-maize amylopectin. *Carbohydrate Research, 212*, 245-251.

Bertoft, E., Boyer, C., Manelius, R., & Avall, A. K. (2000). Observations on the alpha amylolysis pattern of some waxy maize starches from inbred line Ia453. *Cereal Chemistry, 77*, 657-664.

Bertoft, E., & Koch, K. (2000). Composition of chains in waxy-rice starch and its structural units. *Carbohydrate Polymers, 41*, 121-132.

Bertoft, E. (2004). On the nature of categories of chains in amylopectin and their connection to the super helix model. *Carbohydrate Polymers, 57*, 211-224.

Bertoft, E. (2007). Composition of building blocks in clusters from potato amylopectin. *Carbohydrate Polymers, 70*, 123-136.

Bertolini, A. C., Souza, E., Nelson, J. E. & Huber, K. C. (2003). Composition and reactivity of A- and B- type starch granules of normal, partial waxy, and waxy wheat. *Cereal Chemistry, 80*, 544-549.

Bhave, M. R., Lawrence, S., Barton, C., & Hannah, L. C. (1990). Identification and molecular characterization of *shrunken-2* cDNA clones of maize. *Plant Cell, 2*, 581-588.

Biliaderis, C. G., Grant, D. R. & Vose, J. R. (1981). Structural characterization of legume starches. II. Studies on acid treated starches. *Cereal Chemistry, 58,* 502–507.

Bird, A. R., Flory, C., Davies, D. A., Usher, S., & Topping, D. L. (2004). A novel barley cultivar (Himalaya 292) with a specific gene mutation in starch synthase lla raises large bowel starch and short-chain fatty acids in rats. *Journal of Nutrition, 134*, 831-835.

Bjorck, I., Nyman, M., Pedersen, B., Siljestrom, M., Asp, N. G., & Eggum, B. O. (1987). Formation of enzyme resistant starch during autoclaving of wheat-starch - studies *in vitro* and in vivo. *Journal of Cereal Science, 6*, 159-172.

Blanshard, J. M. V. (1987). Starch granule structure and function: A physicochemical approach. In T. Gaillard (Ed.), *Starch Properties and Potential, Critical Reports on Applied Chemistry* (pp. 16-54). New York: John Wiley & Sons.

Blauth, S., Yao, Y., Klucinec, J., Shannon, J., Thompson, D., & Guilitinan, M. (2001). Identification of mutator insertional mutants of starch-branching enzyme 2a in corn. *Plant Physiology, 125,* 1396-1405.

Blauth, S., Kim, K., Klucinec, J., Shannon, J., Thompson, D., & Guiltinan, M. (2002). Identification of mutator insertional mutants of starch-branching enzyme 1 (Sbe1) in *Zea mays* L. *Plant Molecular Biology, 48,* 287-297.

Blazek, J., Salman, H., Rubio, A. L., Gilbert, E., Hanley, T., & Copeland, L. (2009). Structural characterization of wheat starch granules differing in amylose content and functional characteristics. *Carbohydrate Polymers, 75*, 705-711.

Blazek, J., & Copeland, L. (2010). Amylolysis of wheat starches. I. Digestion kinetics of starches with varying functional properties. *Journal of Cereal Science, 51,* 265-270.

Blennow, A., Bay-Smidt, A., & Bauer, R. (2001). Amylopectin aggregation as a function of starch phosphate content studied by size exclusion chromatography and on-line refractive index and light scattering. *International Journal of Biological Macromolecules, 28*, 409-420.

Blennow, A., Bay-Smidt, A., Leonhardt, P., Bandsholm, O., & Madsen, M. (2003). Starch paste stickiness is a relevant native starch selection criterion for wet-end paper manufacturing. *Starch/Stärke, 55,* 381-389.

Boyer, C., Shannon, J., Garwood, D., & Creech, R. (1976). Changes in starch granule size and amylose percentage during kernel development in several *Zea mays* L. genotypes. *Cereal Chemistry, 53,* 327-337.

Boyer, C., & Preiss, J. (1981). Evidence for independent genetic-control of the multiple forms of maize endosperm branching enzymes and starch synthases. *Plant Physiology, 67,* 1141-1145.

Briarty, L. G., Hughes, C. E. & Evers, A. D. (1979). The developing endosperm of wheat – a stereological study. *Annals of Botany, 44,* 641-645.

Brown, I. (2004). Applications and uses of resistant starch. *Journal of AOAC International, 87,* 727-732.

Bugaut, M. & Bentéjac, M. (1993). Biological effects of short-chain fatty acids in nonruminant mammals. *Annual Reviews of Nutrition, 13,* 217-241.

Buléon, A., Gallant, D. J., Bouchet, B., Mouille, G., D'Hulst, C., Kossmann, J. & Ball, S. (1997). Starches from A to C. *Chlamydomonas reinhardtii* as a model microbial system to investigate the biosynthesis of the plant amylopectin crystal. *Plant Physiology, 115,* 949-957.

Buléon, A., Colonna, P., Planchot, V., & Ball, S. (1998). Starch granules: Structure and biosynthesis. *International Journal of Biological Macromolecules, 23,* 85-112.

Buonocore, V., Petrucci, T., & Silano, V. (1977). Wheat protein inhibitors of alpha-amylase. *Phytochemistry, 16,* 811-820.

Burton, R., Bewley, J. D., Smith, A. M., Bhattacharyya, M. K., Tatge, H., Ring, S., Bull, V., Hamilton, W. D. O., & Martin, C. (1995). Starch branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development. *Plant Journal, 7,* 3-15.

Burton, R., Zhang, X., Hrmova, M., & Fincher, G. (1999). A single limit dextrinase gene is expressed both in the developing endosperm and in germinated grains of barley. *Plant Physiology, 119,* 859-871.

Burton, R., Jenner, H., Carrangis, L., Fahy, B., Fincher, G. B., Hylton, C., Laurie, D. A., Parker, M., Waite, D., Wegen, S., Verhoeven, T., & Denyer, K. (2002). Starch granule initiation and growth are altered in barley mutants that lack isoamylase activity. *Plant Journal, 31,* 97-112.

Bustos, R., Fahy, B., Hylton, C. M., Seale, R., Nebane, N. M., Edwards, A., Martin, C., & Smith, A. M. (2004). Starch granule initiation is controlled by a heteromultimeric isoamylase in potato tubers. *Proceedings of the National Academy of Sciences of the United States of America, 101,* 2215-2220.

Butrim, S. M., Litvyak, V. V., & Moskva, V. V. (2009). A study of physicochemical properties of extruded starches of varied biological origin. *Russian Journal of Applied Chemistry, 82,* 1195-1199.

Buttriss, J. L., & Stokes, C. S. (2008). Dietary fibre and health: an overview. *Nutrition Bulletin, 33,* 186-200.

Buttrose, M. S. (1960). Submicroscopic development and structure of starch granules in cereal endosperms. *Journal of Ultrastructure Research, 4,* 231-257.

Buttrose M. S. (1962). The influence of environment on the shell structure of starch granules. *The Journal of Cell Biology, 14,* 159-167.

Campbell, J. M., Bauer, L. L., Fahey, G. C., Jr., Hogarth, A.J.C.L., Wolf, B.W. & Hunter, D.E. (1997). Selected fructooligosaccharide (1-kestose, nystose, and 1F-β fructofuranosylnystose) composition of foods and feeds. *Journal of Agricultural and Food Chemistry, 45,* 3076-3082.

Cao, H., Imparl-Radosevich, J., Guan, H., Keeling, P., James, M., & Myers, A. (1999). Identification of the soluble starch synthase activities of maize endosperm. *Plant Physiology, 120,* 205-215.

Cao, Y-n., Hu, W-g., Wang, C-s. (2012a). Relationship among the key enzymatic activities involved in starch synthesis and amylopectin chain distributions in developing wheat grain. *African Journal of Biotechnology, 11,* 805-814.

Cao, Y. N., Hu, W. G. & Wang, C. S. (2012b). Expression profiles of genes involved in starch synthesis in non-waxy and waxy wheat. *Russian Journal of Plant Physiology, 59,* 632-639.

Carciofi, M., Blennow, A., Jensen, S. L., Shaik, S. S., Henriksen, A., Buléon, A., Holm, P. B. & Hebelstrup, K. H. (2012). Concerted suppression of all starch branching enzyme genes in barley produces amylose-only starch granules. *BMC Plant Biology, 12*, 223-238.

Cardoso, M. B. & Westfahl Jr., H. (2010). On the lamellar width distributions of starch. *Carbohydrate Polymers, 81*, 21-28.

Carman, J., Bishop, D., & Hess, J. (1996). Carbohydrates, minerals and free amino acids in *Triticum aestivum* L. kernels during early embryony. *Journal of Plant Physiology, 149*, 714-720.

Carman, J.G. & Bishop, D.L. (2004). Diurnal $O_2$ and carbohydrate levels in wheat kernels during embryony. *Journal of Plant Physiology, 161*, 1003-1010.

Chao, S., Sharp, P. J., Worland, A. J., Warham, E. J., Koebner, R. M. D. & Gale, M. D. (1989). RFLP-based genetic maps of wheat homeologous group 7 chromosomes. *Theoretical and Applied Genetics, 78*, 495-504.

Chapman, V., Miller, T. E. & and Riley, R. (1976). Equivalence of the A genome of bread wheat and that of *Triticum urartu*. *Genetical Research, 27*, 69–76.

Cheng, C., Mu, J., Farkas, I., Huang, D., Goebl, M., & Roach, P. (1995). Requirement of the self-glucosylating initiator proteins Glg1p and Glg2p for glycogen accumulation in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology, 15*, 6632-6640.

Chibbar, R.N., & Chakraborty, M. (2005). Waxy Wheat. E. Abdel-Aaal, and P. Wood (Eds.), *Speciality grains for food and feed* (pp.143-165). St. Paul, MN: American Association of Cereal Chemists Inc.

Chibbar, R. N., Ganeshan, S., & Båga, M. (2007). *In planta* novel starch synthesis. In. P. Ranalli (Ed.), *Improvement of Crop Plants for Industrial End Uses* (pp. 181-208). Springer.

Chibbar, R. N., Ambigaipalan, P., & Hoover, R. (2010). Molecular diversity in pulse seed starch and complex carbohydrates and its role in human nutrition and health. *Cereal Chemistry, 87*, 342–352.

Cho, M., Wong, L., Marx, C., Jiang, W., Lemaux, P., & Buchanan, B. (1999). Overexpression of thioredoxin h leads to enhanced activity of starch debranching enzyme (pullulanase) in barley grain. *Proceedings of the National Academy of Sciences of the United States of America, 96*, 14641-14646.

Chojecki, A. J. S., Bayliss, M. W. & Gale, M. D. (1986). Cell production and DNA accumulation in the wheat endosperm, and their association with grain weight. *Annals of Botany, 58*, 809-817.

Chung, O. K. & Ohm, J. B. (1997). Wheat lipids as a quality determinant. In J. L. Steele and O.K. Chung (Eds.), *First International Wheat Quality Conference Proceedings* (pp. 83-100). Manhattan, KS: Grain Industry Alliance.

Chung, H. J., Lim, H. S., & Lim, S. T. (2006). Effect of partial gelatinization and retrogradation on the enzymatic digestion of waxy rice starch. *Journal of Cereal Science, 43*, 353-359.

Chung, H-J., Liu, Q., Huang, R., Yin, Y. & Li, A. (2010). Physicochemical properties and *in vitro* starch digestibility of cooked rice from commercially available cultivars in Canada. *Cereal Chemistry, 87,* 297-304.

Cognata, U. L., Willmitzer, L., & Mullerrober, B. (1995). Molecular-cloning and characterization of novel isoforms of potato ADP-glucose pyrophosphorylase. *Molecular & General Genetics, 246,* 538-548.

Colleoni, C., Myers, A. M. & James, M. G. (2003). One- and two- dimensional native PAGE activity gel analyses of maize endosperm proteins reveal functional interactions between specific starch metabolizing enzymes. *Journal of Applied Glycoscience, 50,* 207-212.

Commuri, P., & Keeling, P. (2001). Chain-length specificities of maize starch synthase I enzyme: Studies of glucan affinity and catalytic properties. *Plant Journal, 25,* 475-486.

Craig, J., Lloyd, J. R., Tomlinson, K., Barber, L., Edwards, A., Wang, T. L., Martin, C., Hedley, C. L., & Smith, A. M. (1998). Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in pea embryos. *Plant Cell, 10,* 413-426.

Crowe, T. C., Seligman, S. A., & Copeland, L. (2000). Inhibition of enzymic digestion of amylose by free fatty acids *in vitro* contributes to resistant starch formation. *Journal of Nutrition, 130,* 2006-2008.

Dai, Z. (2010). Activities of enzymes involved in starch synthesis in wheat (*Triticum aestivum* L.) grains differing in starch content. *Russian Journal of Plant Physiology, 57,* 74-78.

Dale, E. M., & Housley, T. L. (1986). Sucrose synthase activity in developing wheat endosperms differing in maximum weight. *Plant Physiology, 82,* 7-10.

Dang, J. M. C., Braet, F. & Copeland, L. (2006). Nanostructural analysis of starch components by atomic force microscopy. *Journal of Microscopy, 224,* 181-186.

Dauvillée, D., Colleoni, C., Shaw, E., Mouille, G., D'Hulst, C., Morell, M., Samuel, M. S., Bouchet, B., Gallant, D. J., Sinskey, A., & Ball, S. (1999). Novel, starch-like polysaccharides are synthesized by an unbound form of granule-bound starch synthase in glycogen-accumulating mutants of *Chlamydomonas reinhardtii*. *Plant Physiology, 119,* 321-329.

Davis, J. P., Supatcharee, N., Khandelwal, R. L., Chibbar, R. N. (2003). Synthesis of Novel Starches in *Planta*: Opportunities and Challenges. *Starch/Stärke, 55,* 107-120.

Debiton, C., Bancel, E., Chambon, C., Rhazi, L. & Branlard, G. (2010). Effect of the three *waxy* null alleles on enzymes associated to wheat starch granules using proteomic approach. *Journal of Cereal Science, 52,* 466-474.

Delcour, J. A., Bruneel, C., Derde, L. J., Gomand, S. V., Pareyt, B., Putseys, J. A., Wilderjans, E., & Lamberts, L. (2010). Fate of starch in food processing: From raw materials to final food products. *Annual Review of Food Science and Technology, 1,* 87-111.

Delrue, B., Fontaine, T., Routier, F., Decq, A., Wieruszeski, J., Koornhuyse, N. V. D., Maddelein, M. –L., Fournet, B., & Ball, S. (1992). Waxy *Chlamydomonas reinhardtii* - monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin. *Journal of Bacteriology, 174*, 3612-3620.

Demeke, T., Hucl, P., Nair, R., Nakamura, T., & Chibbar, R. N. (1997). Evaluation of Canadian and other wheats for waxy proteins. *Cereal Chemistry, 74*, 442-444.

Demeke, T., Hucl, P., Abdel-Aal, E., Båga, M., & Chibbar, R. N. (1999). Biochemical characterization of the wheat waxy A protein and its effect on starch properties. *Cereal Chemistry, 76*, 694-698.

Demeke, T., Hucl, P., & Chibbar, R. N. (2000). Frequent absence of GBSS 1 B isoprotein in endosperm starch of Canadian wheat cultivars. *Starch/Stärke, 52*, 349-352.

Dengate, H., & Meredith, P. (1984). Variation in size distribution of starch granules from wheat grain. *Journal of Cereal Science, 2*, 83-90.

Denyer, K., & Smith, A. (1992). The purification and characterization of the 2 forms of soluble starch synthase from developing pea embryos. *Planta, 186*, 609-617.

Denyer, K., Hylton, C. M., Jenner, C. F., & Smith, A. M. (1995). Identification of multiple isoforms of soluble and granule-bound starch synthase in developing wheat endosperm. *Planta, 196*, 256-265.

Denyer, K., Clarke, B., Hylton, C., Tatge, H., & Smith, A. (1996a). The elongation of amylose and amylopectin chains in isolated starch granules. *Plant Journal, 10*, 1135-1143.

Denyer, K., Dunlap, F., Thorbjornsen, T., Keeling, P., & Smith, A. M. (1996b). The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial. *Plant Physiology, 112*, 779-785.

Denyer, K., Waite, D., Edwards, A., Martin, C. & Smith, A. M. (1999a). Interaction with amylopectin influences the ability of granule-bound starch synthase I to elongate malto oligosaccharides. *Biochemical Journal, 342*, 647–653.

Denyer, K., Waite, D., Motawia, S., Moller, B., & Smith, A. (1999b). Granule-bound starch synthase I in isolated starch granules elongates malto-oligosaccharides processively. *Biochemical Journal, 340*, 183-191.

Denyer, K., Johnson, P., Zeeman, S., & Smith, A. (2001). The control of amylose synthesis. *Journal of Plant Physiology, 158*, 479-487.

Dian, W., Jiang, H. & Wu, P. (2005). Evolution and expression analysis of starch synthase III and IV in rice. *Journal of Experimental Botany, 56*, 623-632.

Dinges, J., Colleoni, C., Myers, A., & James, M. (2001). Molecular structure of three mutations at the maize sugary1 locus and their allele-specific phenotypic effects. *Plant Physiology, 125*, 1406-1418.

Dinges, J., Colleoni, C., James, M., & Myers, A. (2003). Mutational analysis of the pullulanase type debranching enzyme of maize indicates multiple functions in starch metabolism. *Plant Cell, 15*, 666-680.

Dry, I., Smith, A., Edwards, A., Bhattacharyya, M., Dunn, P., & Martin, C. (1992). Characterization of cDNAs encoding 2 isoforms of granule-bound starch synthase which show differential expression in developing storage organs of pea and potato. *Plant Journal, 2*, 193-202.

Dubcovsky, J., & Dvořák, J. (2007). Genome plasticity a key factor in the success of polyploidy wheat under domestication. *Science, 316*, 1862-1866.

Dubois, M., Geddes, W.F. & Smith, F. (1960). The carbohydrates of the Gramineae. X. A quantitative study of the carbohydrates of wheat germ. *Cereal Chemistry, 37*, 557-567.

Dubreil, L., Compoint, J., & Marion, D. (1997). Interaction of puroindolines with wheat flour polar lipids determines their foaming properties. *Journal of Agricultural and Food Chemistry, 45*, 108-116.

Duffus, C. (1992). Control of starch biosynthesis in developing cereal-grains. *Biochemical Society Transactions, 20*, 13-18.

Dvořák, J., Luo, M., Yang, Z., & Zhang, H. (1998). The structure of the *Aegilops tauschii* genepool and the evolution of hexaploid wheat. *Theoretical and Applied Genetics, 97*, 657-670.

Edwards, A., Fulton, D. C., Hylton, C. M., Jobling, S. A., Gidley, M., Rossner, U., Martin, C. & Smith, A. M. (1999). A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. *Plant Journal, 17*, 251-261.

Eerlingen, R. C., Deceuninck, M. & Delcour, J.A. (1993a). Enzyme-resistant starch. II. Influence of amylose chain length on resistant starch formation. *Cereal Chemistry, 70*, 345–50

Eerlingen, R., Crombez, M., & Delcour, J. (1993b). Enzyme-resistant starch .I. quantitative and qualitative influence of incubation-time and temperature of autoclaved starch on resistant starch formation. *Cereal Chemistry, 70*, 339-344.

Ellis, R. P., Cochrane, M. P., Dale, M. F. B., Duffus, C. M., Lynn, A., Morrison, I. M., Prentice, R. D. M., Swanston, J. S., & Tiller, S. (1998). Starch production and industrial use. *Journal of the Science of Food and Agriculture, 77*, 289-311.

Emes, M. J., Bowsher, C. G., Hedley, C., Burrell, M. M., Scrase-Field, E. S. F., & Tetlow, I. J. (2003). Starch synthesis and carbon partitioning in developing endosperm. *Journal of Experimental Botany, 54*, 569-575.

Englyst, H. N., Kingman, S. M., & Cummings, J. H. (1992). Classification and measurement of nutritionally important starch fractions. *European Journal of Clinical Nutrition, 46,* S33-S50.

Escarpa, A., Gonzalez, M. C., Morales, M. D., & SauraCalixto, F. (1997). An approach to the influence of nutrients and other food constituents on resistant starch formation. *Food Chemistry, 60,* 527-532.

Espositio, F., Napolitano, A., Fogliano, V., Arlotti, G., Bonifati, A. M., & Vitale, D. (2005). Antioxidant activity and dietary fiber in durum wheat bran by-products. *Food Research International, 38,* 1167-1173.

Evers, A. D. (1971). Scanning electron microscopy of wheat starch. III. Granule development in the endosperm. *Starch/Stärke, 23,* 157-160.

Evers, A. D. (1973). The size distribution among starch granules in wheat endosperm. *Starch/Stärke, 25,* 303–304.

Fannon, J., Hauber, R., & Bemiller, J. (1992). Surface pores of starch granules. *Cereal Chemistry, 69,* 284-288.

Feldman, M. (2001). The origin of cultivated wheat. In A. P. Bonjean, and W. J. Angus (Eds.), *The World Wheat Book* (pp. 1–56). Paris: Lavoisier Tech & Doc.

Feldman, M., & Levy, A. (2012). Genome evolution due to allopolyploidization in wheat. *Genetics, 192,* 763-774.

Ferguson, L., Tasman-Jones, C., Englyst, H., & Harris, P. (2000). Comparative effects of three resistant starch preparations on transit time and short-chain fatty acid production in rats. *Nutrition and Cancer, 36,* 230-237.

Fernandez, M. R., DePauw, R. M., Knox, R.E., Clarke, J. M., McCaig, T. N., & McLeod, J. G. (1998). AC Crystal red spring wheat. *Canadian Journal of Plant Science, 78,* 307-310.

Fettke, J., Hejazi, M., Smirnova, J., Hoechel, E., Stage, M., & Steup, M. (2009). Eukaryotic starch degradation: Integration of plastidial and cytosolic pathways. *Journal of Experimental Botany, 60,* 2907-2922.

Fifield, C., Weaver, R., & Hayes, J. (1950). Bread loaf volume and protein content of hard red spring wheats. *Cereal Chemistry, 27,* 383-390.

Fincher, G. B. (1989). Molecular and cellular biology associated with endosperm mobilization in germinating cereal-grains. *Annual Review of Plant Physiology and Plant Molecular Biology, 40,* 305-346.

Flipse, E., Suurs, L., Keetels, C., Kossmann, J., Jacobsen, E., & Visser, R. (1996). Introduction of sense and antisense cDNA for branching enzyme in the amylose-free potato mutant leads to physico-chemical changes in the starch. *Planta, 198,* 340-347.

Fox, S. L., Townley-Smith, T. F., Kolmer, J., Harder, D., Gaudet, D. A., Thomas, P. L., Gilbert, J., & Noll, J. S. (2007). AC Splendor hard red spring wheat. *Canadian Journal of Plant Science, 87,* 883-887.

Franco, C. M. L., Ciacco, C. F. & Tavares, D. Q. (1998). The structure of waxy corn starch: Effect of granule size. *Starch/Stärke, 50,* S 193-198.

Franco, C., Wong, K., Yoo, S., & Jane, J. (2002). Structural and functional characteristics of selected soft wheat starches. *Cereal Chemistry, 79*, 243-248.

Fredriksson, H., Silverio, J., Andersson, R., Eliasson, A., & Aman, P. (1998). The influence of amylose and amylopectin characteristics on gelatinization and retrogradation properties of different starches. *Carbohydrate Polymers, 35*, 119-134.

Frei, M., Siddhuraju, P., & Becker, K. (2003). Studies on the *in vitro* starch digestibility and the glycemic index of six different indigenous rice cultivars from the Philippines. *Food Chemistry, 83*, 395-402.

French, A. & Zaslow, B. (1972). Conformation of the "V" amylose helix. *Journal of the Chemical Society, Chemical Communications*, 41-42.

French, D. (1984). In R. L. Whistler, J. N. BeMiller, and J. F. *Paschall* (Eds.), *Starch*: *Chemistry and Technology* (pp. 183-247). New York: Academic Press.

Fu, Y., Ballicora, M., Leykam, J., & Preiss, J. (1998). Mechanism of reductive activation of potato tuber ADP-glucose pyrophosphorylase. *Journal of Biological Chemistry, 273*, 25045-25052.

Fujita, N., & Taira, T. (1998). A 56-kDa protein is a novel granule-bound starch synthase existing in the pericarps, aleurone layers, and embryos of immature seed in diploid wheat (*Triticum monococcum* L.). *Planta, 207*, 125-132.

Fujita, N., Yoshida, M., Asakura, N., Ohdan, T., Miyao, A., Hirochika, H., & Nakamura, Y. (2006). Function and characterization of starch synthase I using mutants in rice. *Plant Physiology, 140,* 1070-1084.

Furukawa, K., Tagaya, M., Tanizawa, K., & Fukui, T. (1993). Role of the conserved lys X-gly gly sequence at the adp-glucose-binding site in *Escherichia coli* glycogen- synthase. *Journal of Biological Chemistry, 268*, 23837-23842.

Gallant, D., Bouchet, B., Buléon, A., & Perez, S. (1992). Physical characteristics of starch granules and susceptibility to enzymatic degradation. *European Journal of Clinical Nutrition, 46,* S3-S16.

Gallant, D., Bouchet, B., & Baldwin, P. (1997). Microscopy of starch: Evidence of a new level of granule organization. *Carbohydrate Polymers, 32*, 177-191.

Ganeshan, S., Drinkwater, J. M., Repellin, A., & Chibbar, R. N. (2010). Selected carbohydrate metabolism genes show coincident expression peaks in grains of *in vitro*-cultured immature

spikes of wheat (*Triticum aestivum* L.). *Journal of Agricultural and Food Chemistry, 58*, 4193-4201.

Gao, M., Wanat, J., Stinard, P. S., James, M. G., & Myers, A. M. (1998). Characterization of dull1, a maize gene coding for a novel starch synthase. *Plant Cell, 10*, 399-412.

Gao, M., & Chibbar, R. N. (2000). Isolation, characterization, and expression analysis of starch synthase IIa cDNA from wheat (*Triticum aestivum* L.). *Genome, 43*, 768-775.

Gautier, M-F., Aleman, M-E., Guirao, A., Marion, D. & Joudrier, P. (1994). *Triticum aestivum* puroindolines, two basic cystine-rich seed proteins: cDNA sequence analysis and developmental gene expression. *Plant Molecular Biology, 25*, 43-57.

Geera, B. P., Nelson, J. E., Souza, E. & Huber, K. C. (2006). Granule-bound starch synthase I (GBSSI) gene effects related to soft wheat flour/ starch characteristics and properties. *Cereal Chemistry, 83*, 544-550.

Genkina, N. K., Wikman, J., Bertoft, E., & Yuryev, V. P. (2007). Effects of structural imperfection on gelatinization characteristics of amylopectin starches with A- and B- type crystallinity. *Biomacromolecules, 8*, 2329-2335.

Genschel, U., Abel, G., Lorz, H., & Lutticke, S. (2002). The sugary-type isoamylase in wheat: Tissue distribution and subcellular localisation. *Planta, 214*, 813-820.

Gerard, C., Planchot, V., Colonna, P., & Bertoft, E. (2000). Relationship between branching density and crystalline structure of A- and B-type maize mutant starches. *Carbohydrate Research, 326*, 130-144.

Gernat, C., Radosta, S., Damaschun, G., & Schierbaum, F. (1990). Supramolecular structure of legume starches revealed by X-ray-scattering. *Starch/Stärke, 42*, 175-178.

Gidley, M. J. & Bociek, S. M. (1988). C-13/MAS NMR studies of amylose inclusion complexes, cyclodextrins, and the amorphous phase of starch granules – relationships between glycosidic linkage conformation and solid-state C-13 chemical shifts. *Journal of the American Chemical Society, 110*, 3820–3829.

Gidley, M. J. (1992). In G. O. Phillips, P. A. Williams, & D. J. Wedlock (Eds.), *Gums and Stabilizers in the Food Industry* (pp. 87-92). Oxford: Oxford University Press.

Gidley, M., Cooke, D., Darke, A., Hoffmann, R., Russell, A., & Greenwell, P. (1995). Molecular order and structure in enzyme-resistant retrograded starch. *Carbohydrate Polymers, 28*, 23-31.

Giroux, M. J., & Hannah, L. C. (1994). ADP-glucose pyrophosphorylase in *shrunken-2* and *brittle-2* mutants of maize. *Molecular & General Genetics, 243*, 400-408.

Giroux, M., Shaw, J., Barry, G., Cobb, B., Greene, T., Okita, T., & Hannah, L. (1996). A single gene mutation that increases maize seed weight. *Proceedings of the National Academy of Sciences of the United States of America, 93*, 5824-5829.

Goggin, D., & Setter, T. (2004). Fructosyltransferase activity and fructan accumulation during development in wheat exposed to terminal drought. *Functional Plant Biology, 31*, 11-21.

Gomez-Casati, D., & Iglesias, A. (2002). ADP-glucose pyrophosphorylase from wheat endosperm. Purification and characterization of an enzyme with novel regulatory properties. *Planta, 214*, 428-434.

Goñi, I., GarciaAlonso, A., & SauraCalixto, F. (1997). A starch hydrolysis procedure to estimate glycemic index. *Nutrition Research, 17*, 427-437.

Graybosch, R. A. (1998). Waxy wheats: origin, properties and prospects. *Trends in Food Science and Technology, 9*, 135-142.

Greenwell, P., & Schofield, J. (1986). A starch granule protein associated with endosperm softness in wheat. *Cereal Chemistry, 63*, 379-380.

Grimaud, F., Rogniaux, H., James, M. G., Myers, A. M., & Planchot, V. (2008). Proteome and phosphoproteome analysis of starch granule-associated proteins from normal maize and mutants affected in starch biosynthesis. *Journal of Experimental Botany, 59*, 3395-3406.

Gromova, I., & Celis, J.E. (2006). Protein detection in gels by silver staining: A procedure compatible with mass-spectroscopy. In J. E. Celis, N. Carter, T. Hunter, K. Simons, J. V. Small, & D. Shotton (Eds.), *Cell Biology: A Laboratory Handbook* (pp. 1-8). Elsevier: Academic Press.

Gross, G. G. (1981). Phenolic acids. In P K. Stumpf & E. E. Conn (Eds.), *The Biochemistry of Plants – A Comprehensive Treatise* (pp. 301-316). New York: Academic Press.

Guan, H., & Preiss, J. (1993). Differentiation of the properties of the branching isozymes from maize (*Zea mays*). *Plant Physiology, 102*, 1269-1273.

Guan, H., & Keeling, P. (1998). Starch biosynthesis: Understanding the functions and interactions of multiple isozymes of starch synthase and branching enzyme. *Trends in Glycoscience and Glycotechnology, 10*, 307-319.

Guillon, F., Larŕe, C., Petipas, F., Berger, A., Moussawi, J., Rogniaux, H., Santoni, A., Saulnier, L., Jamme, F., Miquel, M., Lepiniec, L. & Dubreucq, B. (2011). A comprehensive overview of grain development in *Brachypodium distachyon* variety Bd21. *Journal of Experimental Botany, 63*, 739-755.

Hanashiro, I., Abe, J-i. & Hizukuri, S. (1996). A periodic distribution of the chain length of amylopectin as revealed by high-performance anion-exchange chromatography. *Carbohydrate Research, 283*, 151-159.

Hannah, L. C., & Nelson, O. E., Jr. (1976). Characterization of ADP-glucose pyrophosphorylase from *shrunken-2* and *brittle-2* mutants of maize. *Biochemical Genetics, 14*, 547-560.

Hannah, L. C., & James, M. (2008). The complexities of starch biosynthesis in cereal endosperms. *Current Opinion in Biotechnology, 19*, 160-165.

Harris, P.J., Chavan, R.R. & Ferguson, L.R. (2005). Production and characterisation of two wheat-bran fractions: An aleurone-rich and a pericarp-rich fraction. *Molecular Nutrition and Food Research, 49,* 536-545.

Hendriks, J., Kolbe, A., Gibon, Y., Stitt, M., & Geigenberger, P. (2003). ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plant species. *Plant Physiology, 133,* 838-849.

Hennen-Bierwagen, T. A., Liu, F., Marsh, R. S., Kim, S., Gan, Q., Tetlow, I. J., Emes, M. J., James, M. G., & Myers, A. M. (2008). Starch biosynthetic enzymes from developing maize endosperm associate in multisubunit complexes. *Plant Physiology, 146,* 1892-1908.

Hennen-Bierwagen, T. A., Lin, Q., Grimaud, F., Planchot, V., Keeling, P. L., James, M. G., & Myers, A. M. (2009). Proteins from multiple metabolic pathways associate with starch biosynthetic enzymes in high molecular weight complexes: A model for regulation of carbon allocation in maize amyloplasts. *Plant Physiology, 149,* 1541-1559.

Henry, R. J. (1987). Pentosan and (1-3), (1-4)-β-glucan concentrations in endosperm and wholegrain of wheat, barley, oats and rye. *Journal of Cereal Science, 6,* 253-258.

Henry, R., & Saini, H. (1989). Characterization of cereal sugars and oligosaccharides. *Cereal Chemistry, 66,* 362-365.

Hermansson, A., & Svegmark, K. (1996). Developments in the understanding of starch functionality. *Trends in Food Science & Technology, 7,* 345-353.

Heun, M., SchaferPregl, R., Klawan, D., Castagna, R., Accerbi, M., Borghi, B., & Salamini, F. (1997). Site of einkorn wheat domestication identified by DNA fingerprinting. *Science, 278,* 1312-1314.

Hill, H., Lee, L. S. & Henry, R. J. (2012). Variation in sorghum starch synthesis genes associated with differences in starch phenotype. *Food Chemistry, 131,* 175-183.

Hirose, T., & Terao, T. (2004). A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). *Planta, 220,* 9-16.

Hizukuri, S., Kaneko, T., & Takeda, Y. (1983). Measurement of the chain-length of amylopectin and its relevance to the origin of crystalline polymorphism of starch granules. *Biochimica Et Biophysica Acta General Subjects, 760,* 188-191.

Hizukuri, S. (1985). Relationship between the distribution of the chain-length of amylopectin and the crystalline-structure of starch granules. *Carbohydrate Research, 141,* 295-306.

Hizukuri, S. (1986). Polymodal distribution of the chain lengths of amylopectins, and its significance. *Carbohydrate Research, 147,* 342-347.

Hofvander, P., Andersson, M., Larsson, C., & Larsson, H. (2004). Field performance and starch characteristics of high-amylose potatoes obtained by antisense gene targeting of two branching enzymes. *Plant Biotechnology Journal, 2,* 311-320.

Hoover, R. (2001). Composition, molecular structure, and physicochemical properties of tuber and root starches: A review. *Carbohydrate Polymers, 45*, 253-267.

Hu, P. S., Zhao, H. J., Duan, Z. Y., Zhang, L. L., & Wu, D. X. (2004). Starch digestibility and the estimated glycemic score of different types of rice differing in amylose contents. *Journal of Cereal Science, 40*, 231-237.

Huang, S., Sirikhachornkit, A., Su, X., Faris, J., Gill, B., Haselkorn, R., & Gornicki, P. (2002). Genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase of the *Triticum*/*Aegilops* complex and the evolutionary history of polyploidy wheat. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 8133-8138.

Hucl, P., & Chibbar, R. N. (1996). Variation for starch concentration in spring wheat and its repeatability relative to protein concentration. *Cereal Chemistry, 73*(6), 756-758.

Hughes, G., & Hucl, P. (1993). CDC Teal hard red spring wheat. *Canadian Journal of Plant Science, 73*, 193-197.

Hung, P. V., Yamamori, M., & Morita, N. (2005). Formation of enzyme-resistant starch in bread as affected by high-amylose wheat flour substitutions. *Cereal Chemistry, 82*, 690-694.

Hung, P. V., Maeda, T., Miskelly, D., Tsumori, R., & Morita, N. (2008). Physicochemical characteristics and fine structure of high-amylose wheat starches isolated from Australian wheat cultivars. *Carbohydrate Polymers, 71*, 656-663.

Hussain, H., Mant, A., Seale, R., Zeeman, S., Hinchliffe, E., Edwards, A., Hylton, C., Bornemann, S., Smith, A. M., Martin, C., & Bustos, R. (2003). Three isoforms of isoamylase contribute different catalytic properties for the debranching of potato glucans. *Plant Cell, 15*, 133-149.

Hutchison, C. B. (1921). Heritable characters of maize VII. Shrunken endosperm. *Journal of Heredity, 12*, 76-83.

Hwang, J-W., Kim, S-K., Lee, J-S. & Kim, I-S. (2005). Gene expression of the biosynthetic enzymes and biosynthesis of starch during rice grain development. *Journal of Plant Biology, 48*, 448-455.

Hylton, C., & Smith, A. (1992). The *rb* mutation of peas causes structural and regulatory changes in ADP-glucose pyrophosphorylase from developing embryos. *Plant Physiology, 99*, 1626-1634.

Iglesias, A. A., Barry, G. F., Meyer, C., Bloksberg, L., Nakata, P. A., Greene, T., Laughlin, M. J., Okita, T. W., Kishore, G. M., & Preiss, J. (1993). Expression of the potato-tuber ADP-glucose pyrophosphorylase in *Escherichia coli*. *Journal of Biological Chemistry, 268*, 1081-1086.

Imberty, A., Chanzy, H., Pérez, S., Buléon, A., & Tran, V. (1988a). The double-helical nature of the crystalline part of A-starch. *Journal of Molecular Biology, 201*, 365 378.

Imberty, A., & Perez, S. (1988b). A revisit to the 3-D structure of B-type starch. *Biopolymers, 27*, 1205–1221.

Imparl-Radosevich, J., Li, P., Zhang, L., McKean, A., Keeling, P., & Guan, H. (1998). Purification and characterization of maize starch synthase I and its truncated forms. *Archives of Biochemistry and Biophysics, 353*, 64-72.

Imparl-Radosevich, J. M., Gameon, J. R., McKean, A., Wetterberg, D., Keeling, P. & Guan, H. (2003). Understanding catalytic properties and functions of maize starch synthase isozymes. *Journal of Applied Glycoscience, 50*, 177–182.

Inouchi, N., Hibiu, H., Li, T., Horibata, T., Fuwa, H. & Itani, T. (2005). Structure and properties of endosperm starches from cultivated rice of Asia and other countries. *Journal of Applied Glycoscience, 52*, 239–246.

Ito, T., Saito, K., Sugawara, M., Mochida, K., & Nakakuki, T. (1999). Effect of raw and heat moisture-treated high-amylose corn starches on the process of digestion in the rat digestive tract. *Journal of the Science of Food and Agriculture, 79*, 1203-1207.

Itoh, K., Ozaki, H., Okada, K., Hori, H., Takeda, Y. & Mitsui, T. (2003). Introduction of *wx* transgene into rice *wx* mutants leads to both high- and low- amylose rice. *Plant Cell Physiology, 44*, 473-480.

Jacobsen, S., & Fritz, H. (1996). Filling of poly(lactic acid) with native starch. *Polymer Engineering and Science, 36*, 2799-2804.

Jaiswal, S., Ganeshan, S., Båga, M., & Chibbar, R. N. (2010). *In planta* modification of starch quantity and quality. In Bharat Singh (Ed.), *Industrial crops and uses* (pp. 236-258). Chippenham, U.K.: CABI.

James, M., Robertson, D., & Myers, A. (1995). Characterization of the maize gene *Sugary1*, a determinant of starch composition in kernels. *Plant Cell, 7*, 417-429.

James, M., Denyer, K., & Myers, A. (2003). Starch synthesis in the cereal endosperm. *Current Opinion in Plant Biology, 6*, 215-222.

Jane, J. L., Xu, A., Radosavljevic, M. & Seib, P. A. (1992). Location of amylose in normal starch granules. I. Susceptibility of amylose and amylopectin to cross-linking reagents. *Cereal Chemistry, 69*, 405-409.

Jane, J., & Shen, J. (1993). Internal structure of the potato starch granule revealed by chemical gelatinization. *Carbohydrate Research, 247*, 279-290.

Jane, J., Kasemsuwan, T., Leas S., Zobel, H. & Robyt, J. F. (1994). Anthology of starch granule morphology by scanning electron microscopy. *Starch/Stärke, 46*, 121-129.

Jane, J., Wong, K. S. & McPherson, A. E. (1997). Branch-structure difference in starches of A- and B-type X-ray patterns revealed by their Naegali dextrins. *Carbohydrate Research, 300*, 219-227.

Jane, J., Chen, Y. Y., Lee, L. F., McPherson, A. E., Wong, K. S., Radosavljevic, M. & Kasemsuwan, T. (1999). Effects of amylopectin branch chain length and amylose content on the gelatinization and pasting properties of starch. *Cereal Chemistry, 76,* 629-637.

Jane, J-l. (2007). Structure of starch granules. *Journal of Applied Glycoscience, 54,* 31-36.

Jane, J. L., Maningat, C. C., & Wongsagonsup R. (2010). In B. P. Singh (Ed.), *Industrial Crops and Uses* (pp.). Cambridge, MA: CABI.

Jauhar, P., Rieralizarazu, O., Dewey, W., Gill, B., Crane, C., & Bennett, J. (1991). Chromosome pairing relationships among the A-genome, B-genome, and D-genome of bread wheat. *Theoretical and Applied Genetics, 82,* 441-449.

Jauhar, P. P. (2007). Meiotic resititution in wheat polyhaploids (amphihaploids): A potent evolutionary force. *Journal of Heredity, 98,* 188-193.

Jenkins, J., Cameron, R., & Donald, A. (1993). A universal feature in the structure of starch granules from different botanical sources. *Starch/Stärke, 45,* 417-420.

Jenkins, P., & Donald, A. (1995). The influence of amylose on starch granule structure. *International Journal of Biological Macromolecules, 17,* 315-321.

Jia, L., Zhang, Z., Shu, X., Li, C., & Wu, D. (2007). Starch properties and structure of a wheat mutant high in resistant starch. *The Open Agriculture Journal, 1,* 5-10.

Jiang, H., Campbell, M., Blanco, M. & Jane, J. L. (2010). Characterization of maize amylose extender (*ae*) mutant starches: Part II. Structures and properties of starch residues remaining after enzymatic hydrolysis at boiling-water temperature. *Carbohydrate Polymers, 80,* 1-25.

Jobling, S., Westcott, R., Tayal, A., Jeffcoat, R., & Schwall, G. (2002). Production of a freeze thaw-stable potato starch by antisense inhibition of three starch synthase genes. *Nature Biotechnology, 20,* 295-299.

Jobling, S. (2004). Improving starch for food and industrial applications. *Current Opinion in Plant Biology, 7,* 210-218.

Johnson, P. E., Patron, N. J., Bottrill, A. R., Dinges, J. R., Fahy, B. F., Parker, M. L., Waite, D. N. & Denyer, K. (2003). A low starch barley mutant, *Risø 16*, lacking the cytosolic small subunit of ADP-glucose pyrophosphorylase, reveals the importance of the cytosloic isoform and the identity of the plastidial small subunit. *Plant Physiology, 131,* 684-696.

Jones, J., Reicks, M., Adams, J., Fulcher, G., Weaver, G., Kanter, M., & Marquart, L. (2002). The importance of promoting a whole grain foods message. *Journal of the American College of Nutrition, 21,* 293-297.

Kakefuda, G. & Duke, S. H. (1989). Characterization of pea chloroplast D-enzyme (4-α-d glucanotransferase). *Plant Physiology, 91,* 136–143.

Kasemsuwan, T. & Jane, J. L. (1994). Location of amylose in normal starch granules. II Location of phosphodiester cross-linking revealed by phosphorus-31 nuclear magnetic resonance. *Cereal Chemistry, 71*, 282–287.

Kasemsuwan, T., Jane, J., Schnable, P., Stinard, P., & Robertson, D. (1995). Characterization of the dominant mutant amylose-extender (ael-5180) maize starch. *Cereal Chemistry, 72*, 457-464.

Keeling, P. L., Wood, J. R., Tyson, R. H., & Bridges, I. G. (1988). Starch biosynthesis in developing wheat-grain - evidence against the direct involvement of triose phosphates in the metabolic pathway. *Plant Physiology, 87*, 311-319.

Kendall, C., Emam, A., Augustin, L., & Jenkins, D. (2004). Resistant starches and health. *Journal of AOAC International, 87*, 769-774.

Kequan, Z., & Liangli, Y. (2004). Antioxidant properties of bran extracts from trego wheat grown at different locations. *Journal of Agricultural Food Chemistry, 52*, 1112-1117.

Kim, M., & Lee, S. (2002). Characteristics of crosslinked potato starch and starch-filled linear low-density polyethylene films. *Carbohydrate Polymers, 50*, 331-337.

Kim, W., Johnson, J., Graybosch, R., & Gaines, C. (2003). Physicochemical properties and end use quality of wheat starch as a function of waxy protein alleles. *Journal of Cereal Science, 37*, 195-204.

Kim, H-S., & Huber, K. C. (2010). Physicochemical properties and amylopectin fine structures of A- and B-type granules of waxy and normal soft wheat starch. *Journal of Cereal Science, 51*, 256-264.

Kleczkowski, L., Villand, P., Luthi, E., Olsen, O., & Preiss, J. (1993a). Insensitivity of barley endosperm adp-glucose pyrophosphorylase to 3-phosphoglycerate and orthophosphate regulation. *Plant Physiology, 101*, 179-186.

Kleczkowski, L., Villand, P., Preiss, J., & Olsen, O. (1993b). Kinetic mechanism and regulation of ADP-glucose pyrophosphorylase from barley (*Hordeum vulgare*) leaves. *Journal of Biological Chemistry, 268*, 6228-6233.

Knight, M., Harn, C., Lilley, C., Guan, H., Singletary, G., Mu-Forster, C., Wasserman, B. P., & Keeling, P. (1998). Molecular cloning of starch synthase I from maize (W64) endosperm and expression in *Escherichia coli*. *Plant Journal, 14*, 613-622.

Knudsen, K. (1997). Carbohydrate and lignin contents of plant materials used in animal feeding. *Animal Feed Science and Technology, 67*, 319-338.

Koizumi, K., Fukuda, M. & Hizukuri, S. (1991). Estimation of the distributions of chain length of amylopectins by high-performance liquid chromatography with pulsed amperometric detection. *Journal of Chromatography, 585*, 233-238.

Kolbe, A., Tiessen, A., Schluepmann, H., Paul, M., Ulrich, S., & Geigenberger, P. (2005). Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP

glucose pyrophosphorylase. *Proceedings of the National Academy of Sciences of the United States of America, 102,* 11118-11123.

Kong, X., Bertoft, E., Bao, J., & Corke, H. (2008). Molecular structure of amylopectin from amaranth starch and its effect on physicochemical properties. *International Journal of Biological Macromolecules, 43,* 377-382.

Kong, X., Corke, H., & Bertoft, E. (2009). Fine structure characterization of amylopectins from grain amaranth starch. *Carbohydrate Research, 344,* 1701-1708.

Kooistra, E. (1962). On the differences between smooth and three types of wrinkled peas. *Euphytica, 11,* 357-373.

Koornhuyse, N. V., Libessart, N., Delrue, B., Zabawinski, C., Decq, A., Iglesias, A., Carton, A., Preiss, J., & Ball, S. (1996). Control of starch composition and structure through substrate supply in the monocellular alga *Chlamydomonas reinhardtii*. *Journal of Biological Chemistry, 271,* 16281-16287.

Koroteeva, D. A., Kiseleva, V. I., Krivandin, A. V., Shatalova, O. V., Blaszczak, W., Bertoft, E., Piyachomkwan, K., & Yuryev, V. P. (2007). Structural and thermodynamic properties of rice starches with different genetic background. Part 2. Defectiveness of different supramolecular structures in starch granules. *International Journal of Biological Macromolecules, 41,* 534-547.

Korus, J., Tomasik, P., & Lii, C. (2003). Microcapsules from starch granules. *Journal of Microencapsulation, 20,* 47-56.

Kötting, O., Kossmann, J., Zeeman, S. C. & Lloyd, J.R. (2010). Regulation of starch metabolism: the age of enlightenment? *Current Opinion in Plant Biology, 13,* 321-329.

Kozlov, S. S., Blennow, A., Krivandin, A. V., & Yuryev, V. P. (2007a). Structural and thermodynamic properties of starches extracted from GBSS and GWD suppressed potato lines. *International Journal of Biological Macromolecules, 40,* 449-460.

Kozlov, S. S., Krivandin, A. V., Shatalova, O. V., Noda, T., Bertoft, E., Fornal, J., & Yuryev, V. P. (2007b). Structure of starches extracted from near-isogenic wheat lines - Part II. Molecular organization of amylopectin clusters. *Journal of Thermal Analysis and Calorimetry, 87,* 575-584.

Kroon, P. A., & Williamson, G. (1999). Hydroxycinnamates in plants and food: Current and future perspectives. *Journal of the Science of Food and Agriculture, 79,* 355-361.

Kubo, A., Rahman, S., Utsumi, Y., Li, Z. Y., Mukai, Y., Yamamoto, M., Ugaki, M., Harada, K., Satoh, H., Konik-Rose, C., Morell, M., & Nakamura, Y. (2005). Complementation of sugary-1 phenotype in rice endosperm with the wheat isoamylase1 gene in supports a direct role for isoamylase1 amylopectin biosynthesis. *Plant Physiology, 137,* 43-56.

Kubo, A., Yuguchi, Y., Takeniasa, M., Suzuki, S., Satoh, H., & Kitamura, S. (2008). The use of micro-beam X-ray diffraction for the characterization of starch crystal structure in rice mutant kernels of waxy, amylose extender, and sugary1. *Journal of Cereal Science, 48,* 92-97.

Kulp, K., & Mattern, P. J. (1973). Some properties of starches derived from wheat of varied maturity. *Cereal Chemistry, 50,* 496-504.

Kusaba-Nakayama, M., Ki, M., Kawada, E., Sato, M., Ikeda, I., Mochizuki, T., & Imaizumi, K. (2001). Intestinal absorbability of wheat allergens, subunits of a wheat alpha-amylase inhibitor, expressed by bacteria. *Bioscience, Biotechnology and Biochemistry, 65,* 2448-2455.

Lafiandra, D., Sestili, F., D'Ovidio, R., Janni, M., Botticella, E., Ferrazzano, G., Silverstri, M., Ranieri, R., & DeAmbrogio, E. (2010). Approaches for modification of starch composition in durum wheat. *Cereal Chemistry, 87,* 28-34.

Laohaphatanaleart, K., Piyachomkwan, K., Sriroth, K., & Bertoft, E. (2010). The fine structure of cassava starch amylopectin. Part 1: Organization of clusters. *International Journal of Biological Macromolecules, 47,* 317-324.

Laudencia-Chingcuanco, D. L., Stamova, B. S., You, F. M., Lazo, G. R., Beckles, D. M. & Anderson, O. D. (2007). Transcriptional profiling of wheat caryopsis development using cDNA microarrays. *Plant Molecular Biology, 63,* 651-668.

Lehmann, U., & Robin, F. (2007). Slowly digestible starch - Its structure and health implications: A review. *Trends in Food Science & Technology, 18,* 346-355.

Leloir, L., Rongined, M. A., & Cardini, C. (1961). Starch and oligosaccharide synthesis from uridine diphosphate glucose. *Journal of Biological Chemistry, 236,* 636-641.

Li, Z., Chu, X., Mouille, G., Yan, L., Kosar-Hashemi, B., Hey, S., Napier, J., Shewry, P., Clarke, B., Appels, R., Morell, M. K., & Rahman, S. (1999). The localization and expression of the class II starch synthases of wheat. *Plant Physiology, 120,* 1147-1155.

Li, J. H., Vasanthan, T., Rossnagel, B. & Hoover, R. (2001). Starch from hull-less barley: I. Granule morphology, composition and amylopectin structure. *Food Chemistry, 74,* 395-405.

Li, J., Vasanthan, T., Hoover, R., & Rossnagel, B. (2004). Starch from hull-less barley: V. *In vitro* susceptibility of waxy, normal, and high-amylose starches towards hydrolysis by alpha amylases and amyloglucosidase. *Food Chemistry, 84,* 621-632.

Li, [Chun-Yan], Feng, C-N., Wang, Y-L., Zhang, R., Guo, W-S., Zhu, X-K. & Peng, Y-X. (2007). Chain length distribution of debranched amylopectin and its relationship with physicochemical properties of starch in different wheat cultivars. *Acta Agronomica Sinica, 33,* 1240-1245.

Li, [Li], Blanco, M., & Jane, J. (2007). Physicochemical properties of endosperm and pericarp starches during maize development. *Carbohydrate Polymers, 67,* 630-639.

Li, L., Jiang, H., Campbell, M., Blanco, M., & Jane, J. (2008). Characterization of maize amylose-extender (ae) mutant starches. Part I: Relationship between resistant starch contents and molecular structures. *Carbohydrate Polymers, 74,* 396-404.

Lim, S., Jane, J., Rajagopalan, S., & Seib, P. (1992). Effect of starch granule size on physical properties of starch-filled polyethylene film. *Biotechnology Progress, 8*, 51-57.

Lin, T., Spilatro, S., & Preiss, J. (1988). Subcellular-localization and characterization of amylases in Arabidopsis leaf. *Plant Physiology, 86*, 251-259.

Lindeboom, N., Chang, P., & Tyler, R. (2004). Analytical, biochemical and physicochemical aspects of starch granule size, with emphasis on small granule starches: A review. *Starch/Stärke, 56*, 89-99.

Liu, Z., Chen, S., Ouyang, Z., Guo, Y., Hu, J. & Li, M. (2001). Study on the chain structure of starch molecules by atomic force microscopy. *Journal of Vacuum Science and Technology B, 19*, 111-114.

Liu, Q., Weber, E., Currie, V. & Yada, R. (2003). Physicochemical properties of starches during potato growth. *Carbohydrate Polymers, 51*, 213-221.

Liu, F., Makhmoudova, A., Lee, E. A., Wait, R., Emes, M. J., & Tetlow, I. J. (2009). The amylose extender mutant of maize conditions novel protein-protein interactions between starch biosynthetic enzymes in amyloplasts. *Journal of Experimental Botany, 60*, 4423-4440.

Liu, P., Guo, W., Jiang, Z., Pu, H., Feng, C., Zhu, X., Peng, Y., Kuang, A. & Little, C. R. (2011). Effects of high temperature after anthesis on starch granules in grains of wheat (*Triticum aestivum* L.). *Journal of Agricultural Science, 149*, 159-169.

Lorberth, R., Ritte, G., Willmitzer, L., & Kossmann, J. (1998). Inhibition of a starch-granule-bound protein leads to modified starch and repression of cold sweetening. *Nature Biotechnology, 16*, 473-477.

Lunn, J. E., Feil, R., Hendriks, J. H. M., Gibon, Y., Morcuende, R., Osuna, D., Scheible, W-R., Carillo, R., Hajirezaei, M-R., & Stitt, M. (2006). Sugar induced increases in trehalose 6-phosphate are correlated with redox activation of ADP-glucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*. *Biochemical Journal, 397*, 139-148.

Luo, M. -C., Yang, Z. -L., You, F. M., Kawahara, T., Waines, J. G., & Dvořák, J. (2007). The structure of wild and domesticated emmer wheat populations, gene flow between them, and the site of emmer domestication. *Theoretical and Applied Genetics, 114*, 947-959.

Maddelein, M. L., Libessart, N., Bellanger, F., Delrue, B., D'Hulst C., Koornhuyse, N. V., Fontaine, T., Wieruszeski, J-M., Decq, A., & Ball, S. (1994). Towards an understanding of the biogenesis of the starch granule. Determination of granule-bound and soluble starch synthase functions in amylopectin synthesis. *Journal of Biological Chemistry, 269*, 25150–25157.

Maeda, I., Kiribuchi, S., & Nakamura, M. (1978). Digestion of barley starch granules by combined action of alpha-amylases and beta-amylases purified from barley and barley malt. *Agricultural and Biological Chemistry, 42*, 259-267.

Maley, J., Asare, E. K., Båga, M., Rossnagel, B. G., Chibbar, R. N., & Sammynaiken, R. (2010). Application of aerosol-spray deposition for determination of fine structure of barley starch using atomic force microscopy. *Starch/Stärke, 62*, 676-685.

Maningat, C. C., & Seib, P. A. (1997). Update on wheat starch and it's uses. *Proceedings of the International Wheat Quality Conference* (pp. 261-284). Manhattan, KS: Grain Industry Alliance.

Manners, D. (1989). Recent developments in our understanding of amylopectin structure. *Carbohydrate Polymers, 11*, 87-112.

Martin, C., & Smith, A. (1995). Starch biosynthesis. *Plant Cell, 7*, 971-985.

Matus-Cadiz, M. A. (1999). *Molecular characterization of waxy mutants in hexaploid wheat*. (Doctoral dissertation). Retrieved from University of Saskatchewan's e-library database.

McCaig, T. N., DePauw, R. M., Clarke, J. M., McLeod, J. G., Fernandez, M. R., & Knox, R. E. (1996). AC Barrie hard red spring wheat. *Canadian Journal of Plant Science, 76,* 337-339.

McCallum J. A., & Walker J. R. L. (1990). O-diphenol oxidase activity, phenolic content and colour of New Zealand wheats, flours, and milling streams. *Journal of Cereal Science, 12*, 83-96.

McCance, R. A. & Lawrence, R. D. (1929). The carbohydrate content of foods. Medical Research Council Special Report Series 135, Her Majesty's Stationery Office: London.

McCleary, B. & Codd, R. (1991). Measurement of (1-3), (1-4)-β-D-glucan in barley and oats: A streamlined enzymic procedure. *Journal of the Science of Food and Agriculture, 55*, 303-312.

McCleary, B., Gibson, T., & Mugford, D. (1997). Measurement of total starch in cereal products by amyloglucosidase-alpha-amylase method: Collaborative study. *Journal of AOAC International, 80*, 571-579.

McDonald, A. M. L., Stark, J. R., Morrison, W. R., & Ellis, R. P. (1991). The composition of starch granules from developing barley genotypes. *Journal of Cereal Science, 13*, 93-112.

McFadden, E., & Sears, E. (1946). The origin of *Triticum spelta* and its free-threshing hexaploid relatives. *Journal of Heredity, 37*, 81-89, 107-116.

McIntire, T. M., & Brant, D. A. (1999). Imaging of carrageenan macrocycles and amylose using noncontact atomic force microscopy. *International Journal of Biological Macromolecules, 26,* 303-310.

McPherson, A. E. & Jane, J. (1999). Physicochemical properties of selected root and tuber starches. *Carbohydrate Polymers, 40*, 57-70.

Mentschel, J., & Claus, R. (2003). Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment. *Metabolism-Clinical and Experimental, 52*, 1400-1405.

Miller, H. E., Rigelhof, F., Marquart, L., Prakash, A., & Kanter, M. (2000). Antioxidant content of whole grain breakfast cereals, fruits and vegetables. *Journal of the American College of Nutrition, 19*, 312S-319S.

Mishkind, M., Raikhel, N. V., Palevitz, B. A., & Keegstra, K. (1982). Immunocytochemical localization of wheat germ agglutinin in wheat. *The Journal of Cell Biology, 92*, 753-764.

Miura, H., & Sugawara, A. (1996). Dosage effects of the three Wx genes on amylose synthesis in wheat endosperm. *Theoretical and Applied Genetics, 93*, 1066-1070.

Miura, H., Araki, E., & Tarui, S. (1999). Amylose synthesis capacity of the three Wx genes of wheat cv. Chinese spring. *Euphytica, 108*, 91-95.

Miura, H., Wickramasinghe, M., Subasinghe, R., Araki, E., & Komae, K. (2002). Development of near-isogenic lines of wheat carrying different null Wx alleles and their starch properties. *Euphytica, 123*, 353-359.

Morell, M. K., Bloom, M., Knowles, V., & Preiss, J. (1987). Subunit structure of spinach leaf ADP-glucose pyrophosphorylase. *Plant Physiology, 85*, 182-187.

Morell, M., Bloom, M., & Preiss, J. (1988). Affinity labeling of the allosteric activator site(s) of spinach leaf ADP-glucose pyrophosphorylase. *Journal of Biological Chemistry, 263*, 633-637.

Morell, M., Rahman, S., Abrahams, S., & Appels, R. (1995). The biochemistry and molecular biology of starch synthesis in cereals. *Australian Journal of Plant Physiology, 22*, 647-660.

Morell, M., Blennow, A., KosarHashemi, B., & Samuel, M. (1997). Differential expression and properties of starch branching enzyme isoforms in developing wheat endosperm. *Plant Physiology, 113*, 201-208.

Morell, M. K., Kosar-Hashemi, B., Cmiel, M., Samuel, M. S., Chandler, P., Rahman, S., Buleon, A., Batey, I. L., & Li, Z. (2003). Barley *sex6* mutants lack starch synthase IIa activity and contain a starch with novel properties. *Plant Journal, 34*, 173-185.

Morris, C. (2002). Puroindolines: The molecular genetic basis of wheat grain hardness. *Plant Molecular Biology, 48*, 633-647.

Morrison, W., & Gadan, H. (1987). The amylose and lipid contents of starch granules in developing wheat endosperm. *Journal of Cereal Science, 5*, 263-275.

Morrison, W. (1988). Lipids in cereal starches - a review. *Journal of Cereal Science, 8*, 1-15.

Morrison, W.R. (1989). Uniqueness of wheat starch. In Y. Pomeranz (Ed.), *Wheat is Unique* (pp. 132-214). St. Paul, MN: Ameican Association of Cereal Chemists.

Mossé, J. (1990). Nitrogen to protein conversion factor for ten cereals and six legumes or oilseeds - A reappraisal of its definition and determination - Variation according to species and to seed protein-content. *Journal of Agricultural and Food Chemistry, 38*, 18-24.

Mouille, G., Maddelein, M., Libessart, N., Talaga, P., Decq, A., Delrue, B., & Ball, S. (1996). Preamylopectin processing: A mandatory step for starch biosynthesis in plants. *Plant Cell, 8*, 1353-1366.

Mu, H., Yu, Y., Wasserman, B., & Carman, G. (2001). Purification and characterization of the maize amyloplast stromal 112-kDa starch phosphorylase. *Archives of Biochemistry and Biophysics, 388*, 155-164.

Mua, J., & Jackson, D. (1997). Fine structure of corn amylose and amylopectin fractions with various molecular weights. *Journal of Agricultural and Food Chemistry, 45*, 3840-3847.

Muhrbeck, P., & Eliasson, A. (1991). Influence of the naturally-occurring phosphate-esters on the crystallinity of potato starch. *Journal of the Science of Food and Agriculture, 55*, 13-18.

Mukerjea, R. & Robyt, J. F. (2005). Starch biosynthesis: the primer nonreducing-end mechanism versus the nonprime reducing-end two-site insertion mechanism. *Carbohydrate Research, 340*, 245-255.

Myers, A., Morell, M., James, M., & Ball, S. (2000). Recent progress toward understanding biosynthesis of the amylopectin crystal. *Plant Physiology, 122*, 989-997.

Nachtergaele, W., & Vannuffel, J. (1989). Starch as stilt material in carbonless copy paper – new developments. *Starch/Stärke, 41*, 386-392.

Nadaud, I., Girousse, C., Debiton, C., Chambon, C., Bouzidi, M. F., Martre, P. & Branlard, G. (2010). Proteomic and morphological analysis of early stages of wheat grain development. *Proteomics, 10*, 2901-2910.

Nakamura, T., Vrinten, P., Hayakawa, K., & Ikeda, J. (1998). Characterization of a granule-bound starch synthase isoform found in the pericarp of wheat. *Plant Physiology, 118*, 451-459.

Nakamura, Y. (1996). Some properties of starch debranching enzymes and their possible role in amylopectin biosynthesis. *Plant Science, 121*, 1-18.

Nakamura, Y. (2002). Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: Rice endosperm as a model tissue. *Plant and Cell Physiology, 43*, 718-725.

Nakamura, Y., Sakurai, A., Inaba, Y., Kimura, K., Iwasawa, N., & Nagamine, T. (2002). The fine structure of amylopectin in endosperm from asian cultivated rice can be largely classified into two classes. *Starch/Stärke, 54*, 117-131.

Nakayama, M., Kikuno, R., & Ohara, O. (2002). Protein-protein interactions between large proteins: Two-hybrid screening using a functionally classified library composed of long cDNAs. *Genome Research, 12*, 1773-1784.

Neuhaus, H. E., Batz, O., Thom, E., & Scheibe, R. (1993). Purification of highly intact plastids from various heterotrophic plant-tissues - analysis of enzymatic equipment and precursor dependency for starch biosynthesis. *Biochemical Journal, 296*, 395-401.

Neuhaus, H. E., & Wagner, R. (2000). Solute pores, ion channels, and metabolite transporters in the outer and inner envelope membranes of higher plant plastids. *Biochimica Et Biophysica Acta-Biomembranes, 1465*, 307-323.

Nielsen, T., Baunsgaard, L., & Blennow, A. (2002). Intermediary glucan structures formed during starch granule biosynthesis are enriched in short side chains, a dynamic pulse labeling approach. *Journal of Biological Chemistry, 277*, 20249-20255.

Nikuni, Z. (1969). Proposed model of starch granule or a starch molecule. *Chori Kagaku, 2*, 6-14.

Nilsson, U., Dahlqvist, A. & Nilsson, B. (1986). Cereal fructosans: Part 2. Characterization and structure of wheat fructosans. *Food Chemistry, 22*, 95-106.

Nishi, A., Nakamura, Y., Tanaka, N., & Satoh, H. (2001). Biochemical and genetic analysis of the effects of *amylose-extender* mutation in rice endosperm. *Plant Physiology, 127*, 459-472.

Nishiyama, Y., Putaux, J., Montesanti, N., Hazemann, J., & Rochas, C. (2010). B -> A allomorphic transition in native starch and amylose spherocrystals monitored by in situ synchrotron X-ray diffraction. *Biomacromolecules, 11*, 76-87.

Noda, T., Isono, N., Krivandin, A. V., Shatalova, O. V., Blaszczak, W., & Yuryev, V. P. (2009). Origin of defects in assembled supramolecular structures of sweet potato starches with different amylopectin chain-length distribution. *Carbohydrate Polymers, 76*, 400-409.

Oates, C. G. (1997). Towards an understanding of starch granule structure and hydrolysis. *Trends in Food Science and Technology,8*, 375-382.

Obert, J. C., Ridley, W. P., Schneider, R. W., Riordan, S. G., Nemeth, M. A., Trujillo, W. A., Breeze, M. L., Sorbet, R., & Astwood, J. D. (2004). The composition of grain and forage from glyphosate tolerant wheat MON 71800 is equivalent to that of conventional wheat (*Triticum aestivum* L.). *Journal of Agricultural and Food Chemistry, 52*, 1375-1384.

Oda, S., & Schofield, J. (1997). Characterisation of friabilin polypeptides. *Journal of Cereal Science, 26*, 29-36.

Okita, T., Greenberg, E., Kuhn, D., & Preiss, J. (1979). Subcellular-localization of the starch degradative and biosynthetic-enzymes of spinach leaves. *Plant Physiology, 64*, 187-192.

Okuda, M., Aramaki, I., Koseki, T., Satoh, H., & Hashizume, K. (2005). Structural characteristics, properties, and *in vitro* digestibility of rice. *Cereal Chemistry, 82*, 361-368.

Olive, M. R., Ellis, R. J., & Schuch, W. W. (1989). Isolation and nucleotide-sequences of cDNA clones encoding ADP-glucose pyrophosphorylase polypeptides from wheat leaf and endosperm. *Plant Molecular Biology, 12*, 525-538.

Olsen, O-A. (2001). Endosperm development: Cellularization and cell fate specification. *Annual Review of Plant Physiology and Plant Molecular Biology, 52*, 233-267.

Olsen, O-A. (2004). Nuclear endosperm development in cereals and *Arabidopsis thaliana. Plant Cell, 16*, S214-S227.

O'Shea, M., Samuel, M., Konik, C., & Morell, M. (1998). Fluorophore-assisted carbohydrate electrophoresis (FACE) of oligosaccharides: Efficiency of labelling and high-resolution separation. *Carbohydrate Research, 307*, 1-12.

Pan, D. & Nelson, O. E. (1984). A debranching enzyme deficiency in endosperms of the Sugary-1 mutants of maize. *Plant Physiology, 74*, 324-328.

Panozzo, J. F. & Eagles, H. A. (1998). Cultivar and environmental effects on quality characters in wheat. I. Starch. *Australian Journal of Agricultural Research, 49*, 757-766.

Park, J. T., & Rollings, J. E. (1994). Effects of substrate branching characteristics on kinetics of enzymatic depolymerization of mixed linear and branched polysaccharides: I. Amylose/ amylopectin α- amylolysis. *Biotechnology and Bioengineering, 44*, 792-800.

Parker, M. (1985). The relationship between A-type and B-type starch granules in the developing endosperm of wheat. *Journal of Cereal Science, 3*, 271-278.

Patron, N., Smith, A., Fahy, B., Hylton, C., Naldrett, M., Rossnagel, B., & Denyer, K. (2002). The altered pattern of amylose accumulation in the endosperm of low-amylose barley cultivars is attributable to a single mutant allele of granule-bound starch synthase I with a deletion in the 5 ' non-coding region. *Plant Physiology, 130*, 190-198.

Patron, N. J., Greber, B., Fahy, B. E., Laurie, D. A., Parker, M. L., & Denyer, K. (2004). The lys5 mutations of barley reveal the nature and importance of plastidial ADP-glc transporters for starch synthesis in cereal endosperm. *Plant Physiology, 135*, 2088-2097.

Peñalvo, J. L., Aldercreutz, H., Haajanen, K. M., & Botting, N. (2005). Quantification of lignans in food using isotope dilution gas chromatography/ mass spectrometry. *Journal of Agricultural Food Chemistry, 53*, 9342-9347.

Peng, M., Gao, M., Abdel-Aal, E., Hucl, P., & Chibbar, R. N. (1999). Separation and characterization of A- and B-type starch granules in wheat endosperm. *Cereal Chemistry, 76*, 375-379.

Peng, M. S., Gao, M., Båga, M., Hucl, P., & Chibbar, R. N. (2000). Starch-branching enzymes preferentially associated with A-type starch granules in wheat endosperm. *Plant Physiology, 124*, 265-272.

Peng, M., Hucl, P. & Chibbar, R. N. (2001). Isolation, characterization and expression analysis of starch synthase I from wheat (*Triticum aestivum* L.). *Plant Science, 161*, 1055-1062.

Perera, C., Lu, Z., Sell, J., & Jane, J. (2001). Comparison of physicochemical properties and structures of sugary-2 cornstarch with normal and waxy cultivars. *Cereal Chemistry, 78*, 249-256.

Pérez, S., Imberty, A., & Scaringe, R. P. (1990). Modeling of interactions of polysaccharide chains. In A. D. French and J. W. Brady (Eds.), Computer *Modelling of Carbohydrate Molecules* (pp. 281-299). Washington, DC: ACS Symposium Series.

Pérez, S., & Bertoft, E. (2010). The molecular structures of starch components and their contribution to the architecture of starch granules: A comprehensive review. *Starch/Stärke, 62*, 389-420.

Peterson, R. F. (1965). *Wheat: Botany, cultivation, and utilization*. New York: Interscience Publishers Inc., (Chapter 2).

Peumans, W. J., Stinissen, H. M., & Carlier, A. R. (1982). A genetic basis for the origin of six different isolectins in hexaploid wheat. *Planta, 154,* 562-567.

Peyron, S., Abecassis, J., Autran, J. -C., & Rouau, X. (2001). Enzymatic oxidative treatments of wheat bran layers: Effects on ferulic acid composition and mechanical properties. *Journal of Agricultural Food Chemistry, 49*, 4694-4699.

Piironen, V., Lampi, A-M., Ekholm, P., Salmenkallio-Marttila, M., & Liukkonen, K-H. (2009). Micronutrients and Phytochemicals in Wheat Grain. In K. Khan & P. R. Shewry (Eds), *Wheat Chemistry and Technology* (pp. 179-222). Minnesota: AACC International Press.

Planchot, V., Colonna, P., Buleon, A and Gallent, D. (1997). Amylolysis of starch granules and α-glucan crystallites. In R. J. Frazier, A. M. Donald, & P. Richmond (Eds.), Starch structure and functionality (pp. 141–152). Cambridge: Royal Society of Chemistry.

Polsky, J. (2012). The continuing epidemics of diet-related disease: Environmental drivers of the modern diet and why governments must get involved. *Health Science Inquiry, 3*, 1-2.

Poulsen, P., & Kreiberg, J. D. (1993). Starch branching enzyme cDNA from *Solanum tuberosum*. *Plant Physiology, 102*, 1053-1054.

Preiss, J., Danner, S., Summers, P. S., Morell, M., Barton, C. R., Yang, L., & Nieder, M. (1990). Molecular characterization of the *brittle-2* gene effect on maize endosperm ADP-glucose pyrophosphorylase subunits. *Plant Physiology, 92*, 881-885.

Preiss, J. (1991). Biology and molecular biology of starch synthesis and its regulation. B. J. Miflin (Ed.).*Oxford Surveys of Plant Molecular and Cell Biology* (pp. 59-114). Oxford, England U. K.: Oxford University Press.

Preiss, J. (1993). Biosynthesis of starch: ADP-glucose pyrophosphorylase, the regulatory enzyme of starch synthesis-structure-function relationships. *Denpan Kogako, 40*, 117-131.

Preiss, J., & Sivak, M. (1996). Starch synthesis in sinks and sources. In E. Zamski, and A. A. Schaffter (Eds.), *Photoassimilate distribution in plants and crops: sink-source relationships* (pp 63-96). New York: Marcel Dekker.

Preiss, J., & Sivak, M. N. (1998). Biochemistry, molecular biology and regulation of starch synthesis. In L. Setlow (Ed.), *Genetic Engineering: Principles and Methods, Vol 20* (pp. 177-223). New York: Plenum Press.

Purna, S. K. G., Miller, R. A., Seib, P. A., Graybosch, R. A. & Shi, Y-C. (2011). Volume, texture, and molecular mechanism behind the collapse of bread made with different levels of hard waxy wheat flours. *Journal of Cereal Science, 54*, 37-43.

Raeker, M., Gaines, C., Finney, P., & Donelson, T. (1998). Granule size distribution and chemical composition of starches from 12 soft wheat cultivars. *Cereal Chemistry, 75*, 721-728.

Rahman, S., Kosar-Hashemi, B., Samuel, M. S., Hill, A., Abbott, D. C., Skerritt, J. H., Preiss, J., Appels, R. & Morell, M. K. (1995). The major proteins of wheat endosperm starch granules. *Australian Journal of Plant Physiology, 22*, 793-803.

Rahman, S., Abrahams, S., Abbott, D., Mukai, Y., Samuel, M., Morell, M., & Appels, R. (1997). A complex arrangement of genes at a starch branching enzyme I locus in the D-genome donor of wheat. *Genome, 40*, 465-474.

Rahman, S., Li, Z., Abrahams, S., Abbott, D., Appels, R., & Morell, M. (1999). Characterisation of a gene encoding wheat endosperm starch branching enzyme-I. *Theoretical and Applied Genetics, 98*, 156-163.

Rahman, S., Li, Z., Batey, I., Cochrane, M., Appels, R., & Morell, M. (2000). Genetic alteration of starch functionality in wheat. *Journal of Cereal Science, 31*, 91-110.

Rahman, S., Regina, A., Li, Z., Mukai, Y., Yamamoto, M., Kosar-Hashemi, B., Abrahams, S., & Morell, M. K. (2001). Comparison of starch-branching enzyme genes reveals evolutionary relationships among isoforms. Characterization of a gene for starch-branching enzyme IIa from the wheat D genome donor *Aegilops tauschii*. *Plant Physiology, 125*, 1314-1324.

Ral, J., Colleoni, C., Wattebled, F., Dauvillée, D., Nempont, C., Deschamps, P., Li, Z., Morell, M. K., Chibbar, R. N., Purton, S., d'Hulst, C., & Ball, S. G. (2006). Circadian clock regulation of starch metabolism establishes GBSSI as a major contributor to amylopectin synthesis in *Chlamydomonas reinhardtii*. *Plant Physiology, 142*, 305-317.

Recondo, E., & Leloir, L. (1961). Adenosine diphosphate glucose and starch synthesis. *Biochemical and Biophysical Research Communications, 6*, 85-88.

Reddy, K. R., Ali, S. Z. & Bhattacharya, K. R. (1993). The fine structure of rice-starch amylopectin and its relation to the texture of cooked rice. *Carbohydrate Polymers, 22*, 267-275.

Regina, A., Bird, A., Topping, D., Bowden, S., Freeman, J., Barsby, T., Kosar-Hashemi, B., Li, Z., Rahman, S., & Morell, M. (2006). High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 3546-3551.

Regina, A., Kosar-Hashemi, B., Ling, S., Li, Z., Rahman, S., & Morell, M. (2010). Control of starch branching in barley defined through differential RNAi suppression of starch branching enzyme IIa and IIb. *Journal of Experimental Botany, 61,* 1469-1482.

Repellin, A., R.B. Nair, M. Båga & R.N. Chibbar, 1997. Isolation of a starch branching enzyme I cDNA from a wheat endosperm library (Accession No. Y12320). *Plant Genetics Register*.

Repellin, A., Båga, M. & Chibbar, R. N. (2001). Characterization of a cDNA encoding a type I starch branching enzyme produced in developing wheat (*Triticum aestivum* L.) kernels. *Journal of Plant Physiology, 158,* 91-100.

Repellin, A., Båga, M., & Chibbar, R. N. (2008). *In vitro* pullulanase activity of wheat (*Triticum aestivum* L.) limit-dextrinase type starch debranching enzyme is modulated by redox conditions. *Journal of Cereal Science, 47,* 302-309.

Riaz, M. (1999). Processing biodegradable packaging material from starches using extrusion technology. *Cereal Foods World, 44,* 705-709.

Riley, R., & Chapman, V. (1958). Genetic control of the cytologically diploid behaviour of hexaploid wheat. *Nature, 182,* 713-715.

Rindlav-Westling, A., Stading, M., & Gatenholm, P. (2002). Crystallinity and morphology in films of starch, amylose and amylopectin blends. *Biomacromolecules, 3,* 84-91.

Roldán, I., Wattebled, F., Lucas, M. M., Delvallé, D., Planchot, V., Jiménez, S., Pérez, R., Ball, S., D'Hulst, C., & Mérida, A. (2007). The phenotype of soluble starch synthase IV defective mutants of *Arabidopsis thaliana* suggests a novel function of elongation enzymes in the control of starch granule formation. *Plant Journal, 49,* 492-504.

Rooney, L. W., & Pflugfelder, R. L. (1986). Factors affecting starch digestibility with special emphasis on sorghum and corn. *Journal of Animal Science, 63,* 1607-1623.

Röper, H. (2002). Renewable raw materials in Europe - industrial utilisation of starch and sugar. *Starch/Stärke, 54*(3-4), 89-99.

Ross, A. B., Shepherd, M. J., Schüpphaus, M., Sinclair, V., Alfaro, B., Kamal-Eldin, A., & Åman, P. (2003). Alkylresorcinols in cereals and cereal products. *Journal of Agricultural Food Chemistry, 51,* 4111-4118.

Ryoo, N., Yu, C., Park, C-S., Baik, M-Y., Park, I. M., Cho, M-H., Bhoo, S. H., An, G., Hahn, T-R., & Jeon, J-S. (2007). Knockout of a starch synthase gene *OsSSIIIa/Flo5* causes white-core floury endosperm in rice (*Oryza sativa* L.). *Plant Cell Reports, 26,* 1083-1095.

Sajilata, M., Singhal, R., & Kulkarni, P. (2006). Resistant starch - A review. *Comprehensive Reviews in Food Science and Food Safety, 5,* 1-17.

Sakulsingharoj, C., Choi, S-B., Hwang, S-K., Edwards, G. E., Bork, J., Meyer, C. R., Preiss, J., & Okita, T. W. (2004). Engineering starch biosynthesis for increasing rice seed weight: The role of the cytoplasmic ADP-glucose pyrophosphorylase. *Plant Science, 167,* 1323-1333.

Salcedo, G., Rodriguez-Loperena, A., & Aragoncillo, C. (1978). Relationships among low MW hydrophobic proteins from wheat endosperm. *Phytochemistry, 17,* 1491-1494.

Salman, H., Blazek, J., Lopez-Rubio, A., Gilbert, E. P., Hanley, T., & Copeland, L. (2009). Structure-function relationships in A and B granules from wehat starches of similar amylose content. *Carbohydrate Polymers, 75,* 420-427.

Sang, Y., Bean, S., Seib, P. A., Pedersen, J. &Shi, Y-C. (2008). Structure and functional properties of Sorghum starches differing in amylose content. *Journal of Agricultural and Food Chemistry, 56,* 6680-6685.

Santelia, D., & Zeeman, S. C. (2011). Progress in Arabidopsis starch research and potential biotechnological applications. *Current Opinion in Biotechnology, 22,* 271-280.

Satoh, H., Nishi, A., Yamashita, K., Takemoto, Y., Tanaka, Y., Hosaka, Y., Sakurai, A., Fujita, N., & Nakamura, Y. (2003). Starch-branching enzyme I-deficient mutation specifically affects the structure and properties of starch in rice endosperm. *Plant Physiology, 133,* 1111-1121.

Schwall, G. P., Safford, R., Westcott, R. J., Jeffcoat, R., Tayal, A., Shi, Y-C., Gidley, M. J., & Jobling, S. A. (2000). Production of very-high-amylose potato starch by inhibition of SBE A and B. *Nature Biotechnology, 18,* 551-554.

Sears, E. (1976). Genetic-control of chromosome-pairing in wheat. *Annual Review of Genetics, 10,* 31-51.

Seilmeier, W., Belitz, H., & Wieser, H. (1991). Separation and quantitative-determination of high-molecular-weight subunits of glutenin from different wheat-varieties and genetic-variants of the variety Sicco. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung, 192*, 124-129.

Seo, B., Kim, S., Scott, M., Singletary, G., Wong, K., James, M., & Myers, A. (2002). Functional interactions between heterologously expressed starch-branching enzymes of maize and the glycogen synthases of brewer's yeast. *Plant Physiology, 128*, 1189-1199.

Sestili, F., Botticella, E., Bedo, Z., Phillips, A., & Lafiandra, D. (2010). Production of novel allelic variation for genes involved in starch biosynthesis through mutagenesis. *Molecular Breeding, 25,* 145-154.

Sestili, F., Botticella, E., Proietti, G., Janni, M., D'Ovidio, R., & Lafiandra, D. (2012). Amylose content is not affected by overexpression of the *Wx-B1* gene in durum wheat. *Plant Breeding, 131,* 700-706.

Shannon, J. C. & Garwood, D. L. (1984). In R. L. Whistler, J. N. BeMiller, and E. F. Paschall (Eds.), Starch: Chemistry and Technology, 2nd edition (pp. 25–86). Orlando, FL: Academic Press.

Shannon, J. C., Pien, F. M., Cao, H. P., & Liu, K. C. (1998). *Brittle-1*, an adenylate translocator, facilitates transfer of extraplastidial synthesized ADP-glucose into amyloplasts of maize endosperms. *Plant Physiology, 117*, 1235-1252.

Shewry, P. R., D'Ovidio, R., Lafiandra, D., Jenkins, J. A., Clare Mills, E. N., & Békés, F. (2009). Wheat grain proteins. In K. Khan & P. R. Shewry (Eds.), *Wheat Chemistry and Technology* (pp. 223-298). Minnesota: AACC International Press.

Shibanuma, K., Takeda, Y., Hizukuri, S., & Shibata, S. (1994). Molecular structures of some wheat starches. *Carbohydrate Polymers, 25,* 111-116.

Shu, X., Jia, L., Gao, J., Song, Y., Zhao, H., Nakamura, Y., & Wu, D. (2007). The influences of chain length of amylopectin on resistant starch in rice (*Oryza sativa* L.). *Starch/Stärke, 59*, 504-509.

Shure, M., Wessler, S., & Fedoroff, N. (1983). Molecular-identification and isolation of the waxy locus in maize. *Cell, 35*, 225-233.

Sikka, V. K., Choi, S. B., Kavakli, I. H., Sakulsingharoj, C., Gupta, S., Ito, H., & Okita, T. W. (2001). Subcellular compartmentation and allosteric regulation of the rice endosperm ADP glucose pyrophosphorylase. *Plant Science, 161*, 461-468.

Silverio, J., Fredriksson, H., Andersson, R., Eliasson, A. -C. & Åman, P. (2000). The effect of temperature cycling on the amylopectin retrogradation of starches with different amylopectin unit-chain length distribution. *Carbohydrate Polymers, 42*, 175-184.

Simmonds, D. & O'Brien, T. (1981). Morphological and biochemical development of the wheat endosperm. Minnesota: American Association of Cereal Chemists.

Singh, N., Singh, S., Isono, N., Noda, T. & Singh, A. M. (2009). Diversity in amylopectin structure, thermal and pasting properties of starches from wheat varieties/lines. *International Journal of Biological Macromolecules, 45*, 298-304.

Singletary, G., Banisadr, R., & Keeling, P. (1997). Influence of gene dosage on carbohydrate synthesis and enzymatic activities in endosperm of starch-deficient mutants of maize. *Plant Physiology, 113*, 293-304.

Sivak, M., Wagner, M., & Preiss, J. (1993). Biochemical-evidence for the role of the waxy protein from pea (*Pisum sativum* L.) as a granule-bound starch synthase. *Plant Physiology, 103*, 1355-1359.

Slattery, C., Kavakli, I., & Okita, T. (2000). Engineering starch for increased quantity and quality. *Trends in Plant Science, 5*, 291-298.

Slavin, J., Jacobs, D., & Marquart, L. (2001). Grain processing and nutrition. *Critical Reviews in Biotechnology, 21*, 49-66.

Smidansky, E., Clancy, M., Meyer, F., Lanning, S., Blake, N., Talbert, L., & Giroux, M. (2002). Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 1724-1729.

Smith, A. M., Denyer, K., & Martin, C. (1997). The synthesis of the starch granule. *Annual Review of Plant Physiology and Plant Molecular Biology, 48,* 65-87.

Smith, A. (2001). The biosynthesis of starch granules. *Biomacromolecules, 2,* 335-341.

Smith, A., Zeeman, S., & Smith, S. (2005). Starch degradation. *Annual Review of Plant Biology, 56,* 73-98.

Smith-White, B. J., & Preiss, J. (1992). Comparison of proteins of ADP-glucose pyrophosphorylase from diverse sources. *Journal of Molecular Evolution, 34,* 449-464.

Sokolov, L., Dominguez-Solis, J., Allary, A., Buchanan, B., & Luan, S. (2006). A redox regulated chloroplast protein phosphatase binds to starch diurnally and functions in its accumulation. *Proceedings of the National Academy of Sciences of the United States of America, 103,* 9732-9737.

Song, Y., & Jane, J. (2000). Characterization of barley starches of waxy, normal, and high amylose varieties. *Carbohydrate Polymers, 41,* 365-377.

Soulaka, A., & Morrison, W. (1985). The amylose and lipid contents, dimensions, and gelatinization characteristics of some wheat starches and their A-granule and B-granule fractions. *Journal of the Science of Food and Agriculture, 36,* 709-718.

Srichuwong, S., Sunarti, T., Mishima, T., Isono, N., & Hisamatsu, M. (2005). Starches from different botanical sources I: Contribution of amylopectin fine structure to thermal properties and enzyme digestibility. *Carbohydrate Polymers, 60,* 529-538.

Stark, J., & Lynn, A. (1992). Starch granules large and small. *Biochemical Society Transactions, 20,* 7-12.

Stebbins, G. (1950). Variation and Evolution in Plants. New York: Columbia Univ. Press.

Stinard, P. S., Robertson, D. S., & Schnable, P. S. (1993). Genetic isolation, cloning, and analysis of a mutator-induced, dominant antimorph of the maize amylose-extender1 locus. *Plant Cell, 5,* 1555-1566.

Stoddard, F. L. (1999). Survey of starch particle-size distribution in wheat and related species. *Cereal Chemistry, 76,* 145-149.

Stoddard, F. L. (2003). Genetics of starch granule size distribution in tetraploid and hexaploid wheats. *Australian Journal of Agricultural Research, 54,* 637-648.

Swinburn, B., Caterson, I., Seidell, J., & James, W. (2004). Diet, nutrition and the prevention of excess weight gain and obesity. *Public Health Nutrition, 7,* 123-146.

Syahariza, Z. A., Sar, A., Hasjim, J., Tizzotti, M. J. & Gilbert, R. G. (2013). The importance of amylose and amylopectin fine structures for starch digestibility in cooked rice grains. *Food Chemistry, 136,* 742-749.

Szydlowski, N., Ragel, P., Raynaud, S., Mercedes Lucas, M., Roldán, I., Montero, M., Muñoz, F. J., Ovecka, M., Bahaji, A., Planchot, V., Pozueta-Romero, J., D'Hulst, C., & Mérida, A. (2009). Starch granule initiation in Arabidopsis requires the presence of either class IV or class III starch synthases. *Plant Cell, 21*, 2443-2457.

Takaha, T., Yanase, M., Okada, S., & Smith, S. (1993). Disproportionating enzyme (4-alpha glucanotransferase - ec 2.4.1.25) of potato - purification, molecular-cloning, and potential role in starch metabolism. *Journal of Biological Chemistry, 268*, 1391-1396.

Takeda, Y., Hizukuri, S., & Juliano, B. (1987). Structures of rice amylopectins with low and high affinities for iodine. *Carbohydrate Research, 168*, 79-88.

Takeda, Y., Shitaozono, T. & Hizukuri S. (1990). Structures of subfractions of corn amylose. *Carbohydrate Research, 199*, 207-214.

Takeda, Y., Guan, H., & Preiss, J. (1993). Branching of amylose by the branching isoenzymes of maize endosperm. *Carbohydrate Research, 240*, 253-263.

Takeda, Y., & Hanashiro, I. (2003). Examination of the structure of amylose and amylopectin by fluorescent labeling of reducing terminal. *Journal of Applied Glycoscience, 50*, 163–166.

Takeda, Y., Shibahara, S., & Hanashiro, I. (2003). Examination of the structure of amylopectin molecules by fluorescent labeling. *Carbohydrate Research, 338*, 471-475.

Tang, H., Ando, H., Watanabe, K., Takeda, Y. & Mitsunaga, T. (2001). Physicochemical properties and structures of large, medium, and small granule starches in fractions of normal barley endosperm. *Carbohydrate Research 330*, 241–248.

Tang, H., Watanabe, K. & Mitsunaga, T. (2002). Characterization of storage starches from quinoa, barley and adzuki seeds. *Carbohydrate Polymers, 49*, 13-22.

Tang, M. C. & Copeland, L. (2007). Investigation of starch retrogradation using atomic force microscopy. *Carbohydrate Polymers, 70*, 1-7.

Teo, M-L., & Small, D. M. (2012). The effect of granule size on the digestibility of wheat starch using an *in vitro* model. *World Academy of Science, Engineering and Technology, 69*, 789-793.

Tester, R., Karkalas, J., & Qi, X. (2004). Starch - composition, fine structure and architecture. *Journal of Cereal Science, 39*, 151-165.

Tetlow, I. J., Davies, E. J., Vardy, K. A., Bowsher, C. G., Burrell, M. M., & Emes, M. J. (2003). Subcellular localization of ADP-glucose pyrophosphorylase in developing wheat endosperm and analysis of the properties of a plastidial isoform. *Journal of Experimental Botany, 54*, 715-725.

Tetlow, I., Morell, M., & Emes, M. (2004a). Recent developments in understanding the regulation of starch metabolism in higher plants. *Journal of Experimental Botany, 55*, 2131-2145.

Tetlow, I. J., Wait, R., Lu, Z., Akkasaeng, R., Bowsher, C. G., Esposito, S., Kosar-Hashemi, B., Morell, M. K., & Emes, M. J. (2004b). Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein protein interactions. *Plant Cell, 16,* 694-708.

Tetlow, I. J., Beisel, K. G., Cameron, S., Makhmoudova, A., Liu, F., Bresolin, N. S., Wait, R., Morell, M. K., & Emes, M. J. (2008). Analysis of protein complexes in wheat amyloplasts reveals functional interactions among starch biosynthetic enzymes. *Plant Physiology, 146,* 1878-1891.

Tetlow, I. J. (2011). Starch biosynthesis in developing seeds. *Seed Science Research, 21,* 5-32.

Themeier, H., Hollmann, J., Neese, U. & Lindhauer, M. G. (2005). Structural and morphological factors influencing the quantification of resistant starch II in starches of different botanical origin. *Carbohydrate Polymers, 61,* 72-79.

Thomas, J. B., DePauw, R. M., Knox, R. E., Czarnecki, E., Campbell, A. B., Nielsen, J., McKenzie, R. I. H., Degenhardt, K. J., & Morrison, R. J. (1997). AC Foremost red spring wheat. *Canadian Journal of Plant Science, 77,* 657-660.

Thorbjørnsen, T., Villand, P., Kleczkowski, L. A., & Olsen, O. A. (1996). A single gene encodes two different transcripts for the ADP-glucose pyrophosphorylase small subunit from barley (*Hordeum vulgare*). *Biochemical Journal, 313,* 149-154.

Thorne, J. H. (1985). Phloem unloading of C-assimilates and N-assimilates in developing seeds. *Annual Review of Plant Physiology and Plant Molecular Biology, 36,* 317-343.

Tiessen, A., Hendriks, J., Stitt, M., Branscheid, A., Gibon, Y., Farre, E., & Geigenberger, P. (2002). Starch synthesis in potato tubers is regulated by post-translational redox modification of ADP-glucose pyrophosphorylase: A novel regulatory mechanism linking starch synthesis to the sucrose supply. *Plant Cell, 14,* 2191-2213.

Tillett, I. J. L., & Bryce, J. H. (1993). The regulation of starch granule size in endosperm of developing barley grains. *Proc. 24th European Brewery Convention Congress* (pp. 45-52). Oslo.

Tjaden, J., Mohlmann, T., Kampfenkel, K., Henrichs, G., & Neuhaus, H. E. (1998). Altered plastidic ATP/ADP-transporter activity influences potato (*Solanum tuberosum* L.) tuber morphology, yield and composition of tuber starch. *Plant Journal, 16*(5), 531-540.

Topping, D., & Clifton, P. (2001). Short-chain fatty acids and human colonic function: Roles of resistant starch and non-starch polysaccharides. *Physiological Reviews, 81,* 1031-1064.

Topping, D. L., Morell, M. K., King, R. A., Li, Z., Bird, A. R. & Noakes, M. (2003). Resistant starch and health – *Himalaya 292*, a novel barley cultivar to deliver benefits to consumers. *Starch/Stärke, 55,* 539-545.

Townley-Smith, T. F., Humphreys, D. G., Czarnecki, E., Lukow, O. M., McCallum, B. M., Fetch, T. G., Gilbert, J. A., Menzies, J. G., & Brown, P. D. (2010). Superb hard red spring. *Canadian Journal of Plant Science, 90,* 347-352.

Tsai, C., & Nelson, O. (1966). Starch-deficient maize mutant lacking adenosine diphosphate glucose pyrophosphorylase activity. *Science, 151*, 341-343.

Turner, J. F. (1969). Starch synthesis and changes in UDP-glucose pyrophosphorylase and ADP glucose pyrophosphorylase in the developing wheat grain. *Australian Journal of Biological Sciences, 22*, 1321-1327.

Tyson, R. H., & Rees, T. ap. (1988). Starch synthesis by isolated amyloplasts from wheat endosperm. *Planta, 175*, 33-38.

Umemoto, T., Nakamura, Y., Satoh, H., & Terashima, K. (1999). Differences in amylopectin structure between two rice varieties in relation to the effects of temperature during grain-filling. *Starch/Stärke, 51*, 58-62.

Umemoto, T., Yano, M., Satoh, H., Shomura, A., & Nakamura, Y. (2002). Mapping of a gene responsible for the difference in amylopectin structure between japonica-type and indica-type rice varieties. *Theoretical and Applied Genetics, 104*, 1-8.

Umemoto, T., & Aoki, N. (2005). Single-nucleotide polymorphisms in rice starch synthase IIa that alter starch gelatinisation and starch association of the enzyme. *Functional Plant Biology, 32*, 763-768.

USDA, (2013) http://www.fas.usda.gov/psdonline/psdreport.aspx?hidReportRetrievalName=BVS&hidReportRetrievalID=750&hidReportRetrievalTemplateID=7 (Accessed on June 4th 2013)

Valetudie, J. –C., Colonna, P., Bouchet, B. & Gallant, D. J. (1993). Hydrolysis of tropical tuber starches by bacteria and pancreatic α-amylases. *Starch/Stärke, 45*, 270-276.

Vanderleij, F., Visser, R., Ponstein, A., Jacobsen, E., & Feenstra, W. (1991). Sequence of the structural gene for granule-bound starch synthase of potato (*Solanum-tuberosum* L) and evidence for a single point deletion in the amf allele. *Molecular & General Genetics, 228*, 240-248.

Vasanthan, T., & Bhatty, R. S. (1996). Physicochemical properties of small-and large- granule starches of waxy, regular and high amylose barleys. *Cereal Chemistry, 72*, 199-207.

Verhoogt, H., Stpierre, N., Truchon, F., Ramsay, B., Favis, B., & Ramsay, J. (1995). Blends containing poly(hydroxybutyrate-co-12-percent-hydroxyvalerate) and thermoplastic starch. *Canadian Journal of Microbiology, 41*, 323-328.

Verma, B., Hucl, P., & Chibbar, R. N. (2008). Phenolic content and antioxidant properties of bran in 51 wheat cultivars. *Cereal Chemistry, 85*, 544-549.

Villand, P., Aalen, R., Olsen, O. A., Luthi, E., Lonneborg, A., & Kleczkowski, L. A. (1992a). PCR amplification and sequences of cDNA clones for the small and large subunits of ADP glucose pyrophosphorylase from barley tissues. *Plant Molecular Biology, 19*, 381-389.

Villand, P., Olsen, O. A., Kilian, A., & Kleczkowski, L. A. (1992b). ADP-glucose pyrophosphorylase large subunit cDNA from barley endosperm. *Plant Physiology, 100*, 1617-1618.

Vrinten, P., & Nakamura, T. (2000). Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues. *Plant Physiology, 122*, 255-263.

Waigh, T., Gidley, M., Komanshek, B., & Donald, A. (2000). The phase transformations in starch during gelatinisation: A liquid crystalline approach. *Carbohydrate Research, 328*, 165-176.

Wang, Y., White, P., Pollak, L., & Jane, J. (1993). Characterization of starch structures of 17 maize endosperm mutant genotypes with Oh43 inbred line background. *Cereal Chemistry, 70*, 171-179.

Wang, Y., Liu, W., & Sun, Z. (2003). Effects of granule size and shape on morphology and tensile properties of LDPE and starch blends. *Journal of Materials Science Letters, 22*, 57-59.

Wang, Z., Chen, X., Wang, J., Liu, T., Liu, Y., Zhao, L., & Wang, G. (2007). Increasing maize seed weight by enhancing the cytoplasmic ADP-glucose pyrophosphorylase activity in transgenic maize plants. *Plant Cell Tissue and Organ Culture, 88*, 83-92.

Wang, S., Yu, J., Jin, F., & Yu, J. (2008). The new insight on ultrastructure of C-type starch granules revealed by acid hydrolysis. *International Journal of Biological Macromolecules, 43*, 216-220.

Wang, L., Xie, B., Shi, J., Xue, S., Deng, Q., Wei, Y., & Tian, B. (2010). Physicochemical properties and structure of starches from Chinese rice cultivars. *Food Hydrocolloids, 24*, 208-216.

Watanabe, T., & French, D. (1980). Structural features of naegeli amylodextrin as indicated by enzymic degradation. *Carbohydrate Research, 84*, 115-123.

Weber, H., Heim, U., Borisjuk, L., & Wobus, U. (1995). Cell-type-specific, coordinate expression of 2 ADP-glucose pyrophosphorylase genes in relation to starch biosynthesis during seed development *of Vicia faba* L. *Planta, 195*, 352-361.

Weber, C., Haugaard, V., Festersen, R., & Bertelsen, G. (2002). Production and applications of biobased packaging materials for the food industry. *Food Additives and Contaminants, 19*, 172-177.

Wei, C., Qin, F., Zhu, L., Zhou, W., Chen, Y., Wang, Y., Gu, M., & Liu, Q. (2010a). Microstructure and ultrastructure of high-amylose rice resistant starch granules modified by antisense RNA inhibition of starch branching enzyme. *Journal of Agricultural and Food Chemistry, 58*, 1224-1232.

Wei, C., Zhang, J., Chen, Y., Zhou, W., Xu, B., Wang, Y., & Chen, J. (2010b). Physicochemical properties and development of wheat large and small starch granules during endosperm development. *Acta Physiologiae Plantarum, 32,* 905-916.

Weidner, S., Amarowicz, R., Karamac, M., & Dabrowski, G. (1999). Phenolic acids in caryopses of two cultivars of wheat, rye, triticale that display different resistance to pre-harvest sprouting. *European Food Research and Technology, 210,* 109-113.

Wickramasinghe, H. A. M., & Miura, H. (2003). Gene dosage effect of the wheat Wx alleles and their interaction on amylose synthesis in the endosperm. *Euphytica, 132,* 303-320.

Wright, H. T., Sandrasegaram, G., & Wright, C. S. (1991). Evolution of a family of N-acetylglucosamine binding proteins containing the disulfide-rich domain of wheat germ agglutinin. *Journal of Molecular Evolution, 33,* 283-294.

Wrigley, C. (1996). Biopolymers - giant proteins with flour power. *Nature, 381,* 738-739.

Wu, H. & Sarko, A. (1978a). The double-helical molecular structure of crystalline A- amylose. *Carbohydrate Research, 61,* 27-40.

Wu, H. & Sarko, A. (1978b). The double-helical molecular structure of crystalline B- amylose. *Carbohydrate Research, 61,* 7-25.

Wu, C., Colleoni, C., Myers, A., & James, M. (2002). Enzymatic properties and regulation of ZPU1, the maize pullulanase-type starch debranching enzyme. *Archives of Biochemistry and Biophysics, 406,* 21-32.

Yamamori, M., Nakamura, T., & Kuroda, A. (1992). Variations in the content of starch-granule-bound protein among several Japnese cultivars of common wheat (*Triticum aestivum* L.). *Euphytica, 64,* 215-219.

Yamamori, M., Fujita, S., Hayakawa, K., Matsuki, J., & Yasui, T. (2000). Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylose. *Theoretical and Applied Genetics, 101,* 21-29.

Yamamori, M., Kato, M., Yui, M., & Kawasaki, M. (2006). Resistant starch and starch pasting properties of a starch synthase IIa-deficient wheat with apparent high amylose. *Australian Journal of Agricultural Research, 57,* 531-535.

Yamamori, M. (2009). Amylose content and starch properties generated by five variant *Wx* alleles for granule-bound starch synthase in common wheat (*Triticum aestivum* L.). *Euphytica, 165,* 607-614.

Yang, J., Zhang, J., Wang, Z., Xu, G. & Zhu, Q. (2004). Activities of key enzymes in sucrose-to starch conversion in wheat grains subjected to water deficit during grain filling. *Plant Physiology, 135,* 1621-1629.

Yao, N., Paez, A. V. & White, P. J. (2009). Structure and function of starch and resistant starch from corn with different doses of mutant *amylose-extender* and *floury-1* alleles. *Journal of Agricultural and Food Chemistry, 57*, 2040-2048.

Yasui, T., Matsuki, J., Sasaki, T., & Yamamori, M. (1996). Amylose and lipid contents, amylopectin structure, and gelatinisation properties of waxy wheat (*Triticum aestivum*) starch. *Journal of Cereal Science, 24*, 131-137.

Yoo, S. H., & Jane, J. L. (2002). Structural and physical characteristics of waxy and other wheat starches. *Carbohydrate Polymers, 49*, 297-305.

Yoshimoto, Y., Takenouchi, T. & Takeda, Y. (2002). Molecular structure and some physicochemical properties of waxy and low-amylose barley starches. *Carbohydrate Polymers, 47*, 159-167.

Yu, Y., Mu, H., Mu-Forster, C., & Wasserman, B. (1998). Polypeptides of the maize amyloplast stroma - stromal localization of starch-biosynthetic enzymes and identification of an 81 kilodalton amyloplast stromal heat-shock cognate. *Plant Physiology, 116*, 1451-1460.

Yu, T. S., Kofler, H., Häusler, R. E., Hille, D., Flügge U-I., Zeeman, S. C., Smith, A. M., Kossmann, J., Llyod, J., Ritte, G., Steup, M., Lue, W-L., Chen, J., & Weber, A. (2001). The Arabidopsis sex1 mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. *Plant Cell, 13*, 1907-1918.

Yun, F., & McDonald, C. E. (1989). Comparison of flavanoids in bran of four classes of wheat. *Cereal Chemistry, 66,* 516-518.

Zeeman, S., Umemoto, T., Lue, W., Au-Yeung, P., Martin, C., Smith, A., & Chen, J. (1998). A mutant of arabidopsis lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. *Plant Cell, 10*, 1699-1711.

Zeeman, S., Smith, S., & Smith, A. (2004). The breakdown of starch in leaves. *New Phytologist, 163*, 247-261.

Zeeman, S.C., Kossmann, J., & Smith, A.M. (2010). Starch: its metabolism, evolution, and biotechnological modification in plants. *Annual Review of Plant Biology, 61,* 209–234.

Zhang, X. L., Colleoni, C., Ratushna, V., Sirghle-Colleoni, M., James, M. G., & Myers, A. M. (2004). Molecular characterization demonstrates that the *Zea mays* gene *sugary2* codes for the starch synthase isoform SSIIa. *Plant Molecular Biology, 54*, 865-879.

Zhang, X. L., Myers, A. M., & James, M. G. (2005). Mutations affecting starch synthase III in Arabidopsis alter leaf starch structure and increase the rate of starch synthesis. *Plant Physiology, 138*, 663-674.

Zhang, G., Ao, Z., & Hamaker, B. R. (2006). Slow digestion property of native cereal starches. *Biomacromolecules, 7*, 3252-3258.

Zhang, G., Ao, Z. & Hamaker, B. R. (2008). Nutritional property of endosperm starches from maize mutants: A parabolic relationship between slowly digestible starch and amylopectin fine structure. *Journal of Agricultural and Food Chemistry, 56,* 4686-4694.

Zhang, C., Jiang, D., Liu, F., Cai, J., Dai, T., & Cao, W. (2010). Starch granules size distribution in superior and inferior grains of wheat is related to enzyme activities and their gene expressions during grain filling. *Journal of Cereal Science, 51,* 226-233.

Zhao, X., & Sharp, P. (1996). An improved 1-D SDS-PAGE method for the identification of three bread wheat 'waxy' proteins. *Journal of Cereal Science, 23,* 191-193.

Zhao, X. C., Batey, I. L., Sharp, P. J., Crosbie, G., Barclay, I., Wilson, R., Morell, M. K., & Appels, R. (1998). A single genetic locus associated with starch granule properties and noodle quality in wheat. *Journal of Cereal Science, 27*, 7-13.

Zhao, X.C., & Sharp, P.J. (1998). Production of all eight genotypes of null alleles at ‘waxy’ loci in bread wheat, *Triticum aestivum* L. *Plant Breeding, 117,* 488-490.

Zhu, Z., Hylton, C., Rossner, U., & Smith, A. (1998). Characterization of starch-debranching enzymes in pea embryos. *Plant Physiology, 118,* 581-590.

Zhu, L-J., Liu, Q-Q., Wilson, J. D. Gu, M-H., & Shi, Y-C. (2011). Digestibility and physicochemical properties of ricr (*Oryza sativa* L.) flours and starches differing in amylose content. *Carbohydrate Polymers, 86,* 1751-1759.

Zobel, H. F. (1992). In R. J. Alexander, and H. F. Zobel (Eds.), Developments in Carbohydrate Chemistry (pp 1-36). St. Paul, MN: American Association of Cereal Chemistry.